Universidade de Aveiro Departamento de Química
Ano 2014

ISABEL MENDES DA SILVA

ANÁLISE DA MATÉRIA ORGÂNICA ASSOCIADA AO SAL MARINHO

Definição de potenciais marcadores moleculares com base na composição volátil e presença de derivados glicosilados e polissacarídeos

ANALYSIS OF THE ORGANIC MATTER ASSOCIATED TO SEA SALT

Definition of potential molecular markers based on the volatile composition and presence of glycosidic derivatives and polysaccharides

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Sílvia Maria da Rocha Simões Carriço, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho à minha família e em especial ao meu marido, pelo incansável apoio.

«Há vários milhares de anos caíram aqui as célebres janelas do palácio do Céu. Ficaram intactas as vidraças nos respectivos caixilhos, porque as janelas caíram sobre a relva verdinha. Hoje são as salinas.»

(José de Almada Negreiros, in Aveiro, primeiras impressões, "Panorama", n.º 1,1941)

o júri

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palavras-chave

Sal marinho, matéria orgânica, potenciais marcadores moleculares, compostos voláteis, cromatografia de gás bidimensional abrangente (GC×GC), material polimérico, polissacarídeos sulfatados, proteína, triacilglicerídeos, fatores ambientais

resumo

O sal marinho é um produto natural que deriva da evaporação da água do mar nas salinas pela ação do vento e da luz solar. Atualmente existe um interesse crescente na proteção e revalorização das salinas, o que está intrinsecamente associado à qualidade do sal, que pode ser avaliada pelas suas características físico-químicas. Estes sistemas construídos pelo homem podem encontrar-se em diferentes áreas geográficas, apresentando diferentes envolventes ambientais. Durante o processo de cristalização, compostos orgânicos provenientes da envolvente ambiental podem ficar incorporados nos cristais de sal, influenciando a sua composição. A matéria orgânica associada ao sal marinho é proveniente de três principais origens: algas, comunidade bacteriana envolvente e atividade antropogénica.

Com base na hipótese de que o sal marinho contém compostos orgânicos associados que podem ser utilizados como marcadores do próprio sal marinho, incluindo as condições ambientais envolventes das salinas, o objetivo desta tese de doutoramento foi identificar estes compostos no sal. Para tal, procedeu-se a: 1) uma caracterização detalhada da composição volátil do sal marinho por microextração em fase sólida do espaço de cabeça combinada com cromatografia em fase gasosa bidimensional abrangente acoplada à espectrometria de massa por tempo de voo (HS-SPME/GC×GC–ToFMS), proporcionando a identificação de potenciais marcadores voláteis; 2) ao desenvolvimento de uma metodologia que permitisse isolar material polimérico potencialmente presente no sal marinho, em quantidades suficientes para a sua caracterização em termos de polissacarídeos e proteína; e 3) à exploração da possível presença de triacilglicerídeos.

A elevada resolução cromatográfica e sensibilidade da GC×GC–ToFMS possibilitou a separação e identificação de um maior número de compostos voláteis do sal marinho, cerca do triplo, em comparação com a cromatografia unidimensional (GC–qMS). Os cromatogramas bidimensionais revelaram a presença de 165 compostos, pertencentes a 11 famílias químicas, o que mostra a complexidade da composição volátil do sal marinho. O perfil cromatográfico em duas dimensões, resultante da separação por volatilidade em ^{1}D e por polaridade em ^{2}D, permite a dispersão dos compostos no espaço cromatográfico bidimensional em que os compostos estruturalmente similares ocupam o mesmo espaço cromatográfico (cromatograma estruturado), o que permitiu maior fiabilidade nas identificações de acordo os tempos de retenção ($^{1}t_R$ e $^{2}t_R$) e espetro de massa.

Os resultados da análise de sal proveniente de dois locais do salgado de Aveiro, recolhido ao longo de três anos, sugerem a perda de compostos voláteis ao longo do tempo de armazenamento do sal.

A partir de sais do Oceano Atlântico provenientes de sete origens geográficas, todos produzidos em 2007, foi possível identificar um conjunto de dez compostos presentes em todos os sais, nomeadamente: 6-metil-5-hepteno-2-ona, 2,2,6-trimetil-ciclo-hexanona, isoforona, cetoisoforona, 5,6-epóxi-β-ionona, di-hidroactinidiolida, 6,10,14-trimetil-2-pentadecanona, 2-metilpropanoato de 3-hidroxi-2,4,4-trimetilpentilo, bis(2-metilpropanoato) de 2,4,4-trimetilpentano-1,3-diilo e 2-etil-1-hexanol. Estes dez compostos foram considerados como potenciais marcadores voláteis do sal marinho. Sete destes compostos são derivados dos carotenoides e os restantes três poderão resultar da integração de compostos provenientes da atividade antropogénica no metabolismo dos organismos marinhos.

Com o presente trabalho foi isolado e caracterizado, pela primeira vez, o material polimérico presente no sal marinho. Foram usados 16 sais do Oceano Atlântico. Foi desenvolvida uma metodologia baseada num processo de diálise, que permitiu isolar material polimérico a partir de sal marinho em quantidades suficientes que permitissem a sua caracterização. O conteúdo de material polimérico isolado a partir dos 16 sais foi, em mediana, 144 mg por kg de sal seco, i.e. 0,014% (m/m). A espetroscopia de infravermelho médio e a termogravimetria revelaram a presença maioritária de polissacarídeos sulfatados no material polimérico do sal marinho e também a presença de proteína. Os polissacarídeos do sal marinho mostraram ser ricos em resíduos de ácido urónico (21 mol%), glucose (18), galactose (15) e fucose (13). O conteúdo em sulfato representou, em mediana, 45 mol%, sendo a mediana do conteúdo em polissacarídeos sulfatados de 461 mg/g de material polimérico, o que representa 66 mg/kg de sal seco. As ligações glicosídicas encontradas indicaram que os resíduos de açúcares maioritários que poderiam conter um ou mais grupos sulfato eram a fucose e a galactose. Este facto leva a propor que os polissacarídeos do sal marinho tenham origem principalmente em algas, tendo em conta a sua composição e abundância. O perfil em aminoácidos do material polimérico dos 16 sais em estudo revelou como resíduos maioritários, em mediana, a alanina (25 mol%), a leucina (14) e a valina (14), que têm em comum a sua hidrofobicidade, sendo a mediana do conteúdo em proteína de 35 mg/g, i.e. 4,9 mg por kg de sal seco.

Para além da fração hidrofóbica volátil, também foram detetados compostos hidrofóbicos não voláteis no sal marinho. Foram isolados triacilglicerídeos por extração com soxhlet, usando *n*-hexano. A composição em resíduos de ácidos gordos revelou como ácido maioritário o ácido palmítico (43 mol%), seguido do esteárico (13), linolénico (13), oleico (12) e linoleico (9). O conteúdo, em mediana, de triacilglicerídeos foi de 1,5 mg por kg de sal seco. Dada a sua composição e abundância, tanto a proteína como os triacilglicerídeos parecem ter origem em macro e microalgas, fitoplacton e cianobactérias.

Apesar da variabilidade resultante do ambiente envolvente das salinas, a presente tese de doutoramento permitiu identificar um perfil em compostos orgânicos característico do sal marinho com base nos compostos voláteis, polissacarídeos, proteína e triacilglicerídeos.

keywords

Sea salt, organic matter, potential molecular markers, volatile compounds, comprehensive two-dimensional gas chromatography (GC × GC), polymeric material, sulfated polysaccharides, triacylglycerides, protein, saltpans surrounding environment

abstract

Sea salt is a natural product obtained from the evaporation of seawater in saltpans due to the combined effect of wind and sunlight. Nowadays, there is a growing interest for protection and re-valorisation of saltpans intrinsically associated to the quality of sea salt that can be evaluated by its physico-chemical properties. These man-made systems can be located in different geographical areas presenting different environmental surroundings. During the crystallization process, organic compounds coming from these surroundings can be incorporated into sea salt crystals, influencing their final composition. The organic matter associated to sea salt arises from three main sources: algae, surrounding bacterial community, and anthropogenic activity.

Based on the hypothesis that sea salt contains associated organic compounds that can be used as markers of the product, including saltpans surrounding environment, the aim of this PhD thesis was to identify these compounds. With this purpose, this work comprised: 1) a deep characterisation of the volatile composition of sea salt by headspace solid phase microextraction combined with comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (HS-SPME/GC×GC–ToFMS) methodology, in search of potential sea salt volatile markers; 2) the development of a methodology to isolate the polymeric material potentially present in sea salt, in amounts that allow its characterisation in terms of polysaccharides and protein; and 3) to explore the possible presence of triacylglycerides.

The high chromatographic resolution and sensitivity of GC×GC–ToFMS enabled the separation and identification of a higher number of volatile compounds from sea salt, about three folds, compared to unidimentional chromatography (GC–qMS). The chromatographic contour plots obtained revealed the complexity of marine salt volatile composition and confirmed the relevance of GC×GC–ToFMS for this type of analysis. The structured bidimentional chromatographic profile arising from ^{1}D volatility and ^{2}D polarity was demonstrated, allowing more reliable identifications.

Results obtained for analysis of salt from two locations in Aveiro and harvested over three years suggest the loss of volatile compounds along the time of storage of the salt.

From Atlantic Ocean salts of seven different geographical origins, all produced in 2007, it was possible to identify a sub-set of ten compounds present in all salts, namely 6-methyl-5-hepten-2-one, 2,2,6-trimethylcyclohexanone, isophorone, ketoisophorone, β-ionone-5,6-epoxide, dihydroactinidiolide, 6,10,14-trimethyl-2-pentadecanone, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate, 2,4,4-trimethylpentane-1,3-diyl bis(2-methylpropanoate), and 2-ethyl-1-hexanol. These ten compounds were considered potential volatile markers of sea salt. Seven of these compounds

are carotenoid-derived compounds, and the other three may result from the integration of compounds from anthropogenic activity as metabolites of marine organisms.

The present PhD work also allowed the isolation and characterisation, for the first time, of polymeric material from sea salt, using 16 Atlantic Ocean salts. A dialysis-based methodology was developed to isolate the polymeric material from sea salt in amounts that allowed its characterisation. The median content of polymeric material isolated from the 16 salts was 144 mg per kg of salt, e.g. 0.014% (w/w). Mid-infrared spectroscopy and thermogravimetry revealed the main occurrence of sulfated polysaccharides, as well as the presence of protein in the polymeric material from sea salt. Sea salt polysaccharides were found to be rich in uronic acid residues (21 mol%), glucose (18), galactose (16), and fucose (13). Sulfate content represented a median of 45 mol%, being the median content of sulfated polysaccharides 461 mg/g of polymeric material, which accounted for 66 mg/kg of dry salt. Glycosidic linkage composition indicates that the main sugar residues that could carry one or more sulfate groups were identified as fucose and galactose. This fact allowed to infer that the polysaccharides from sea salt arise mainly from algae, due to their abundance and composition. The amino acid profile of the polymeric material from the 16 Atlantic Ocean salts showed as main residues, as medians, alanine (25 mol%), leucine (14), and valine (14), which are hydrophobic, being the median protein content 35 mg/g, i.e. 4,9 mg per kg of dry salt.

Beside the occurrence of hydrophobic volatile compounds in sea salt, hydrophobic non-volatile compounds were also detected. Triacylglycerides were obtained from sea salt by soxhlet extraction with *n*-hexane. Fatty acid composition revealed palmitic acid as the major residue (43 mol%), followed by stearic (13), linolenic (13), oleic (12), and linoleic (9). Sea salt triacylglycerides median content was 1.5 mg per kg of dry salt. Both protein and triacylglycerides seem to arise from macro and microalgae, phytoplankton and cyanobacteria, due to their abundance and composition.

Despite the variability resulting from saltpans surrounding environment, this PhD thesis allowed the identification of a sea salt characteristic organic compounds profile based on volatile compounds, polysaccharides, protein, and triacylglycerides.

Publications in the aim of this thesis

Publications in international journals with referees:

Silva, I.; Rocha, S. M., Coimbra, M. A., Marriott, P. J. (2010) Headspace solid-phase microextraction combined with comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry for the determination of volatile compounds from marine salt. Journal of Chromatography A, 1217, 5511-5521.

Silva, I.; Marriott, P. J.; Coimbra, M. A.; Rocha, S. M. (2014) Can volatile organic compounds be markers of sea salt? Submitted.

Silva, I.; Nunes, A.; Rocha, S. M.; Coimbra, M. A. (2014) Characterisation of food grade Atlantic salts according to their triacylglycerides, polysaccharides, and protein content. Submitted.

Silva, I.; Quitério, P.; Maricato, E.; Nunes, C.; Rocha, S. M.; Coimbra, M. A. Characterisation of the highly sulfated polysaccharides from sea salt. Under preparation.

Abstracts of communications in scientific meetings:

Silva, I.; Rocha, S. M.; Coimbra, M. A. (2011) "Extracção e caracterização do material polimérico presente no sal marinho". XXII Encontro Nacional da Sociedade Portuguesa de Química, Braga, Portugal, pp. 69.

Silva, I.; Rocha, S. M.; Coimbra, M. A.; Marriott, P. (2009) "Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry analysis of volatile compounds from marine salt", 6º Encontro Nacional de Cromatografia, Madeira, Portugal, pp. 167.

Silva, I.; Rocha, S. M.; Coimbra, M. A.; Marriott, P. (2008) "Characterisation of the volatile composition of marine salt by comprehensive two-dimensional gas chromatography", ACROSS Symposium on Advances in Separation Science, Tasmania, Australia, pp.105.

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ABBREVIATIONS

18C	18 dos Caramonetes
1D	One dimension
^{1}D	First dimension
$^{1}t_R$	Retention time for the first dimension
2D	Two dimensions
^{2}D	Second dimension
2EH	2-Ethyl-1-hexanol
$^{2}t_R$	Retention time for the second dimension
3HPP	3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate
6MHO	6-Methyl-5-hepten-2-one
AA	Amino acid
Ala	Alanine
Ara	Arabinose
Asn	Asparagine
Asp	Aspartic acid
AV	Aveiro
C14:0	Myristic acid
C15:0	Pentadecylic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
C20:0	Arachidic acid
C22:0	Behenic acid
C23:0	Tricosylic acid
C24:0	Lignoceric acid
C26:0	Cerotic acid
CAR	Carboxen
CD	Cádiz
CG	Coarse Gold
CM	Castro Marim
CW	Carbowax
d_f	Film thickness
DHA	Dihydroactinidiolide

DMSO	Dimethyl sulfoxide
dRib	Deoxyribose
DTG	Derivative thermogravimetry
DVB	Divinylbenzene
FA	Fatty acid
FF	Figueira da Foz
FID	Flame ionization detector
Fuc	Fucose
Gal	Galactose
GC	Gas chromatography
GCa	Grã Caravela
GC×GC	Comprehensive two-dimensional gas chromatography
Glc	Glucose
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HCA	Hierarchical Cluster Analysis
HS	Headspace
I.D.	Internal diameter
IEC	Ion extraction chromatography
Ile	Isoleucine
Leu	Leucine
LMCS	Longitudinally modulated cryogenic system
LP	La Palma Island
Man	Mannose
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
nP	Non-polar
P	Polar
PCA	Principal component analysis
PDMS	Polydimethylsiloxane
PF	Pink Flakes
Phe	Phenylalanine
PJ	Peijota
PM	Polymeric material
PMAA	Partially methylated alditol acetates
Pro	Proline
PUFA	Polyunsaturated fatty acid
qMS	Quadrupole mass spectrometry

Rha	Rhamnose
RI	Retention index
Rib	Ribose
RSD	Relative standard deviation
S	Sal Island
Ser	Serine
SFA	Saturated fatty acid
SPME	Solid phase microextraction
TCA	Trichloroacetic acid
TCH	2,2,6-Trimethylcyclohexanone
TFA	Trifluoroacetic acid
TG	Triacylglyceride
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
Thr	Threonine
TIC	Total ion current
TMPP	2,4,4-Trimethylpentane-1,3-diyl bis(2-methylpropanoate)
ToFMS	Time-of-flight mass spectrometry
TPD	6,10,14-Trimethyl-2-pentadecanone
UA	Uronic acid
Val	Valine
Xyl	Xylose

Thesis Overview

The present thesis begins with an introduction and background (Chapter I) on the subject to be studied, the organic matter associated to sea salt. The chapter starts with a brief description of the sea salt composition and how it is obtained from nature in saltpans. After some historical references, it is shown how important is the revalorisation of the product "sea salt" and survival of the natural and cultural heritage associated to it, and what has been done, particularly in legal terms, to distinguish the salt from natural sources from industrial salt. A description of the saltpans surrounding environment is also presented, followed by a background about the organic matter associated to sea salt, namely what is already known in terms of its volatile fraction. Based on the hypothesis that sea salt contains organic compounds associated to their crystals that can be used as markers of the sea salt itself, including saltpans surrounding environment, and with the aim of identifying these compounds, the main goals of this PhD thesis were established and are presented at the end of Chapter I.

Sea salt samples under study and experimental procedures performed are described in Chapter II.

The results of this PhD thesis along with their discussion are presented in Chapter III. This is divided in four sections, according to the different subjects discussed. The first section (III.A) refers to the development of a methodology to characterise the volatile and semi-volatile compounds of sea salt by headspace solid-phase microextraction (HS-SPME) combined with comprehensive two-dimensional gas chromatography - time-of-flight mass spectrometry (GC×GC–ToFMS). Samples from two saltpans of Aveiro, in Portugal, with diverse locations, obtained over three years (2004, 2005, and 2007) were used in this study. Then, in section III.B, the search of potential volatile markers of sea salt was performed using sea salts from seven origins: France, Portugal, Continental Spain, Canary Islands, and Cape Verde, which were analysed by the HS-SPME/GC×GC–ToFMS developed methodology. The following two sections explore the non-volatile organic fraction of sea salt. Section III.C presents a dialysis-based methodology to isolate polymeric material from sea salt in amounts that allow its characterisation. This section focuses on the analysis and characterisation of polysaccharides, based on polymeric material isolated from 16 Atlantic salts. The last section of this chapter (section III.D) is an exploratory study dedicated to the presence of protein and triacylglycerides in sea salt, in which these two

important groups of biomolecules are characterised for the same 16 Atlantic salts used in section III.C.

The origin of all the organic compounds found in sea salt, volatiles and non-volatiles, was explored based on information found in literature about the surrounding environment of saltpans. This lead to the proposal of the existence of a typical pattern regarding the organic matter associated to sea salt and the presence of potential molecular markers of this natural product.

In summary, **Fig. 0.1** shows the overview scheme of the present PhD thesis, including the samples used, the target, the chemical families analysed, and the outputs.

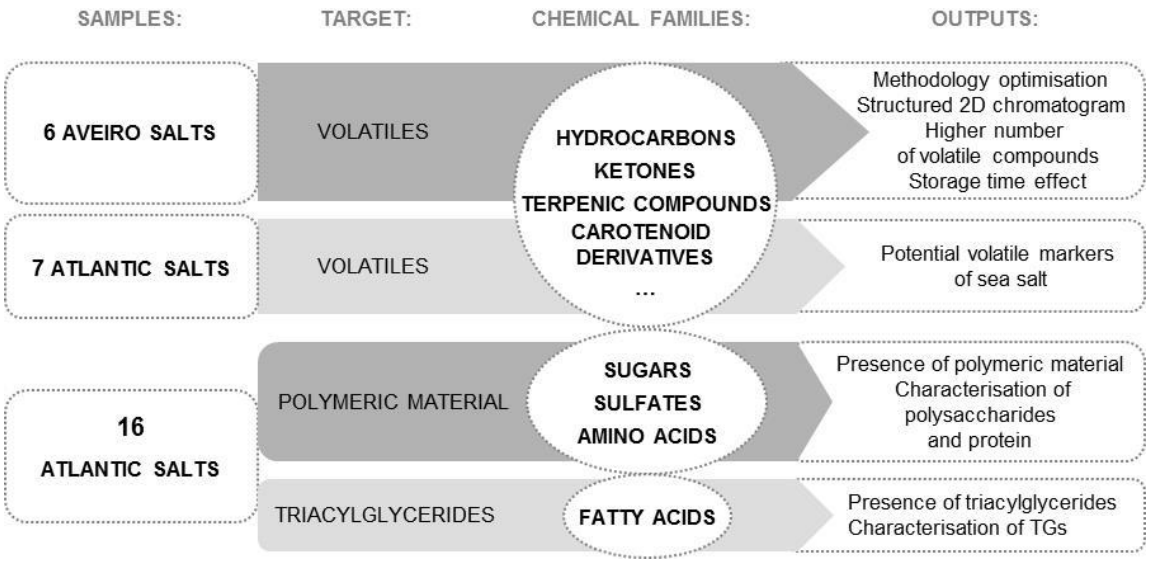

Figure 0.1. Overview scheme of the present PhD thesis.

1.1. SEA SALT

Sea salt is a crystalline mineral primarily composed of sodium chloride (NaCl). Besides NaCl, sea salt contains a small percentage of other components such as salts of calcium (e.g. $CaCO_3$, $CaSO_4$), potassium (e.g. KCl) and magnesium (e.g. $MgCl_2$, $MgSO_4$), trace elements including iodine, iron, and zinc, and also an organic fraction of volatile compounds (Silva *et al.*, 2009; Silva *et al.*, 2010). The crystals of NaCl, present in large majority, have a face-centered cubic lattice with four sodium and four chloride ions per unit cell of the cubic structure (**Fig. 1.1**).

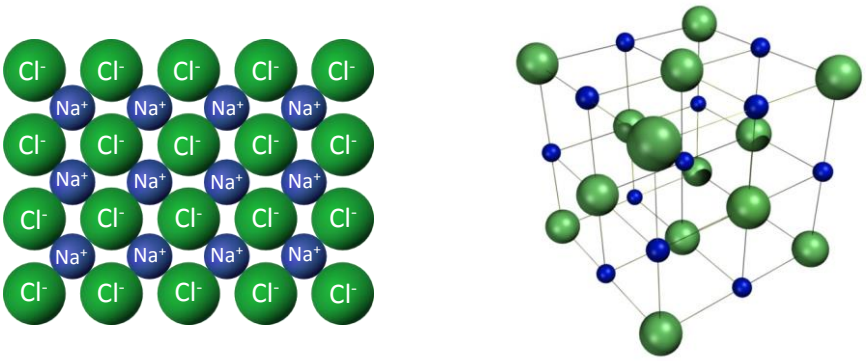

Figure 1.1. Ionic composition and crystalline structure of sodium chloride.
(http://commons.wikimedia.org/wiki/File:NaCl_crystal_structure.png; http://commons.wikimedia.org/wiki/File:Nacl-structure.jpg)

Sea salt may present different granulometries and colours (**Fig. 1.2**), characteristics related with its way of production and geographical origin. For example, crystals of *fleur de sel* are usually smaller than crystals of common sea salt and, depending on the minerals present in seawater, sea salts may present different colours.

Figure 1.2. Physical appearance of different sea salts.
(http://www.maineseasalt.com/)

Since the ocean contains a virtually inexhaustible supply of NaCl (about 3.5%, w/w of salinity), man-made systems were developed to obtain this crystalline mineral from seawater. These systems where salt is produced by the evaporation of seawater due to a combined effect of wind and sunlight are named saltpans (**Fig. 1.3**). In saltpans the seawater circulates by gravity, flowing through different ponds with increasing levels of salinity due to a continuous evaporation. Along the way, decantation of silt and algae occurs. The harvest of the salt is possible when the point of crystallisation is achieved ($s_{(NaCl)}$ = 35.92 g / 100 g of aqueous solution at 25ºC) and the salt crystals precipitate (Burgess, 1978). *Fleur de sel* are the first crystals formed in the seawater surface which are carefully collected before being deposited. This man-made production usually involves minimal but prolongued processing, such as manual washing, natural drying, crushing, and sieving of sea salt.

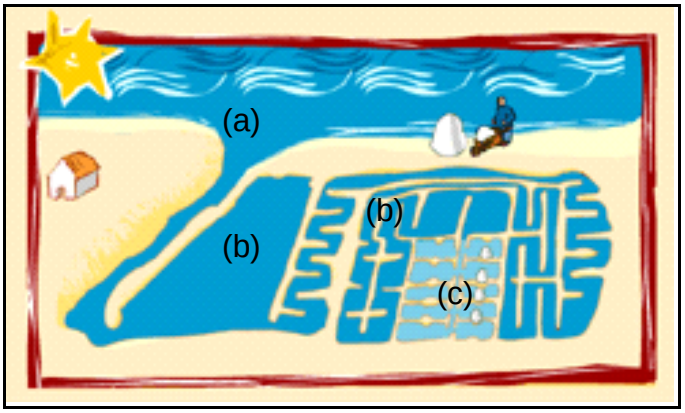

Figure 1.3. Schematic example of the structure of a saltpan: (a) Seawater supply; (b) Concentration ponds; (c) Crystallization ponds

(http://www.aquasel.fr/index.asp?ID=371&idl=381)

Sea salt may also occur naturally in the so-called inland saltpans (**Fig. 1.4**), located in inland places near the coast, where seawater emerges spontaneously forming salt crystals at the surface.

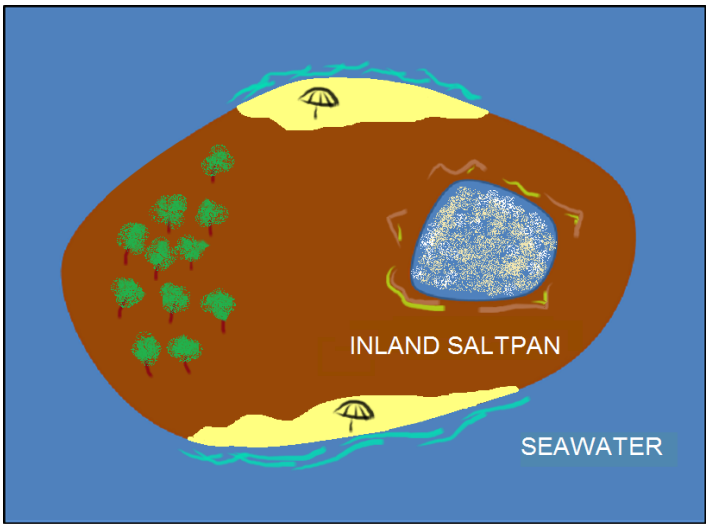

Figure 1.4. Schematic example of an inland saltpan.

Before saltpans became common man-made systems for the production of salt from seawater, this mineral was not so easy to obtain. Given its importance, namely, being essential for life[1], and one of the oldest, most ubiquitous food seasonings that still represents a great importance in food preservation, salt represented a highly valued trade item (**Fig. 1.5**), such that, s*alarium*, the Latin origin of the word 'salary', has the etymology in the name given to the payment in salt to Roman legionnaires.

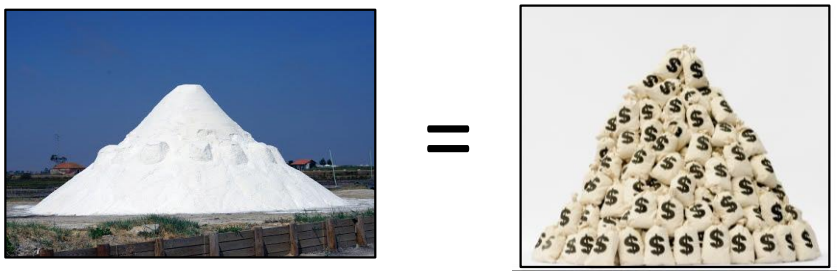

Figure 1.5. Salt, once a highly valued trade item.

(http://www.panoramio.com/photo/81666996?tag=Ria%20de%20Aveiro%20-%20Locais; http://fernandonogueiracosta.files.wordpress.com/2013/02/monte-de-sacos-de-dinheiro.jpg)

In Portugal, Aveiro is one of the regions where it is possible to find active saltpans. Situated in the Vouga river lagoon, these are dedicated to the production of common sea

[1] According to new guidelines issued by the World Health Organization (WHO) of 31 January 2013, adults should consume less than 2000 mg of sodium, or 5 g of salt per day.

salt and also *fleur de sel*. Nevertheless, the number of active saltpans in Aveiro, similarly to what happened in other Portuguese regions, suffered an expressive decrease during the last 60 years, as it is possible to see in **Fig. 1.6**. Between 1956 and 2011 more than 100 saltpans were inactivated. This is related to the fact that from the mid-19th century, the rock salt (halite) obtained from salt mining gained expression. Also, the use of salt as a preserving agent decreased and the improvement of land transportation took place. However, the production of sea salt is still a reality in many different regions of the world. In Portugal, the main sea salt productions are in Figueira da Foz, Alcochete, Ria Formosa, and Castro Marim.

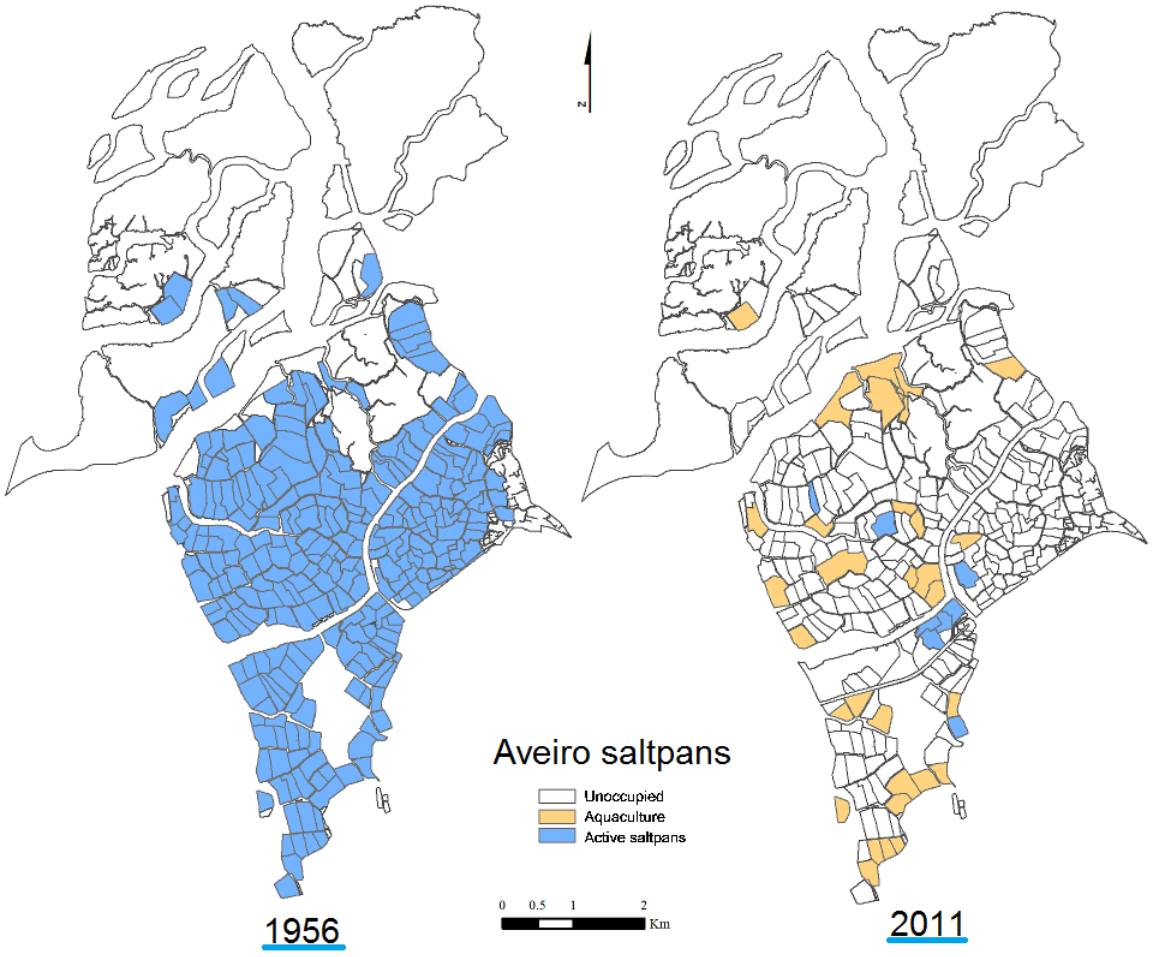

Figure 1.6. Aveiro saltpans active in 1956 and in 2011.

(Adapted from an image provided by the Department of Environment and Planning from University of Aveiro)

Aiming the revalorisation of the product "sea salt" and the survival of the natural and cultural heritage associated to it, a project entitled SAL – Sal do Atlântico -

"Revalorisation of the Atlantic traditional saltpans identity. Recovery and promotion of the biological, economical, and cultural potential of the humid zones from the coast", supported by the European Commission (INTERREG IIIB Programme) was carried on from 2004 to 2007. This was a support for the research on this field, namely in areas such as engineering, biology, and chemistry. Another important step on the valorisation of traditional saltpans and sea salt was the establishment of specific legislation regarding the use of salt for food purposes. In Portugal, the legislation was published in 2007, establishing a legal framework for the production and marketing of salt for food purposes (Decreto-Lei n.º 350/2007). This differentiates sea salt and other forms to obtain salt from natural sources ('*sal tal qual*'), from salt that was submitted to appropriate industrial treatment after its extraction ('*sal tratado*'). Manual washing, natural drying, crushing, and sieving of sea salt are not considered industrial treatment. On the other hand, processes such as cleaning, refining, and enrichment of salt, with iodine for example, are recognized as industrial treatments. All these salts have to contain a minimum of 90% of NaCl, on a dry basis. In the case of '*sal tal qual*' technical standards were established, as well as the characteristics and conditions for the production, marketing, and commercialization of this natural product (Portaria n.º 72/2008).

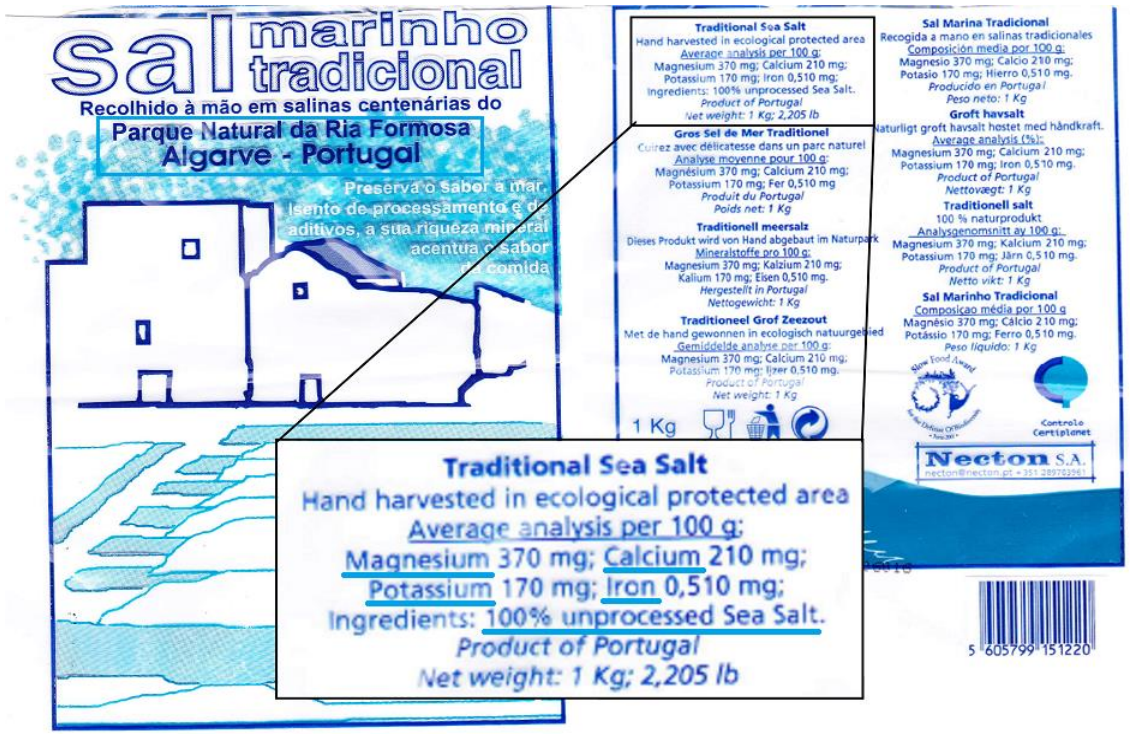

Figure 1.7. Example of a label from a sea salt package (Ria Formosa, Portugal)

For example, sea salt packages usually highlight the local of origin, reinforce the idea of a minimally or even non processed product, and have mentions to the content in some trace elements (**Fig. 1.7**).

1.2. SALTPANS SURROUNDING ENVIRONMENT

Saltpans can be located in geographical areas presenting different environmental surroundings. They are present in estuarine zones, i.e. transition zones between river and sea environments, which may present substantial intertidal habitats with an extensive benthic fauna and flora (McLusky & Elliott, 2007). Saltpans can also be located in non-estuarine zones such as inland saltpans near the sea or seaside saltpans, which can be either arid zones or surrounded by vegetation, in the coast and inland sites of continents and islands. Thus, the surrounding environment of these systems comprise seawater that supplies saltpans, halophilic organisms that usually colonize these hypersaline habitats, vegetation that may be present near saltpans, and the surrounding atmosphere (**Fig. 1.8**).

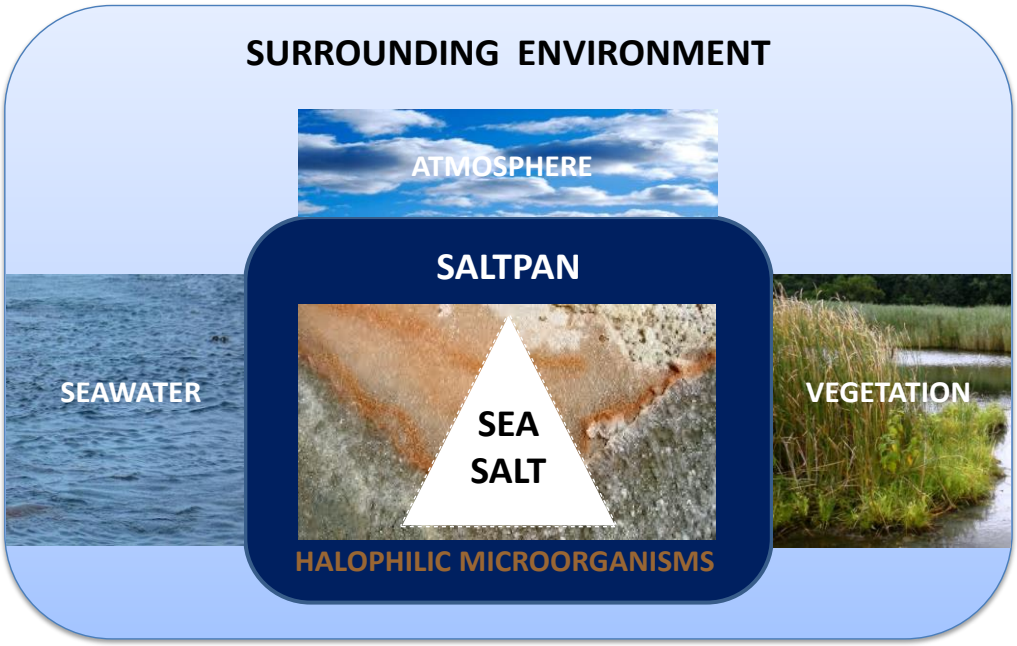

Figure 1.8. Saltpans surrounding environment.

Saltpans are hypersaline habitats where only certain living organisms can survive. There are several previous studies focusing the habitat of hypersaline environments, including plants, algae, or bacterial communities. In these environments it is possible to find halophytes, plants that grow in high salinity waters (Baron, 1979), such as *Salicornia europaea, Halimione portulacoides* (Meziane *et al.*, 1997), *Limoniastrum monopetalum*, and *Spartina densiflora* (Simões *et al.*, 2011); halophilic microalgae, namely of *Dunaliella* spp. such as *Dunaliella salina* (Kaçka & Dönmez, 2008; Donadio *et al.*, 2011); halophilic bacteria, as for example *Lentibacillus salicampi* (Yoon *et al.*, 2002) and *Salirhabdus euzebyi* (Albuquerque *et al.*, 2007), and cyanobacteria (Evans, 1994). In the seawater that surrounds and supplies saltpans, a wide range of marine organisms may be also present, including seaweeds, microalgae, phytoplankton, marine invertebrates, and bacteria, among others. With respect to surrounding vegetation, this can be very diverse, according to the geographical location of the saltpan. Plants such as grass *Dactylis glomerata*, and bushes *Sueda vera* and *Quercus ilex* have already been identified nearby these habitats (Biorede, 2001). The surrounding atmosphere can also interfere with saltpans, since atmospheric components may undergo a deposition process. Another aspect that sometimes may not be disregarded is the presence of human activity or the proximity of saltpans to urban zones. These may involve issues such as boat traffic, aquaculture, proximity to roads or industries, and contamination by sewage.

1.3. SEA SALT ORGANIC MATTER

The inputs of organic matter into the salt matrix arise from the surrounding environment of saltpans. For example, seawater that maintain saltpans carries organic components coming from marine living organisms (Bravo-Linares & Mudge, 2009; Engel & Händel, 2011), such as algae, phytoplankton, marine invertebrates, and microorganisms. This is the case of estuarine zones, sometimes inhabited by large populations of seaweeds. These represent a source of organic compounds that can be released into the media by cell lysis, apoptosis, or exudation (Giani *et al.*, 2005; Urbani *et al.*, 2005; Engel & Händel, 2011). All the surrounding players mentioned in section 1.2 represent a potential contribution to the organic matter associated to sea salt, either in terms of its volatile composition (Evans, 1994),

or/and presence of polymeric material (Jiao *et al.*, 2011) or/and triacylglycerides (van Ginneken *et al.*, 2011), among others.

1.3.1. *Volatile fraction*

In a previous study, a headspace solid-phase microextraction method combined with one-dimensional gas chromatography–quadrupole mass spectrometry (HS-SPME/GC–qMS) methodology was developed to study the volatile composition of sea salt (Silva *et al.*, 2009). This first investigation on volatile compounds of sea salt revealed the presence of volatile and semi-volatile organic compounds distributed over the chemical groups of hydrocarbons, alcohols, phenols, aldehydes, ketones, esters, terpenoids, and norisoprenoids. According to the literature, these compounds arise from three main sources: algae, surrounding bacterial community, and anthropogenic activities (Silva *et al.*, 2009) (**Fig. 1.9**). More recent studies on the volatile composition of sea salt also describe

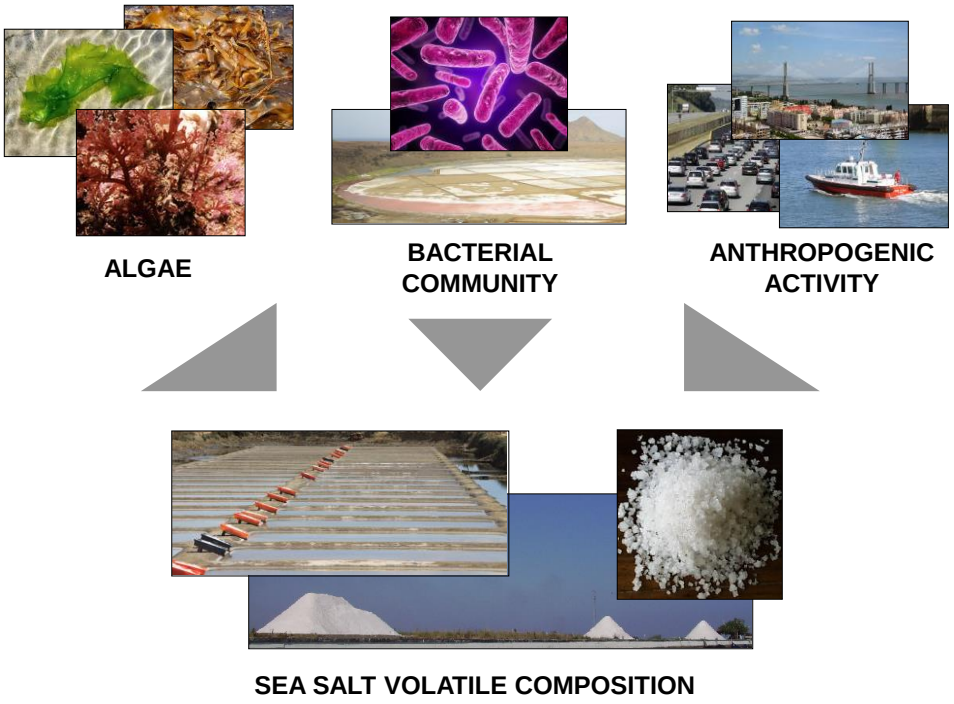

Figure 1.9. Influence of the surrounding environment in the sea salt volatile composition.

these as possible origins (Donadio *et al.*, 2011; Serrano *et al.*, 2011). Namely, the presence of norisoprenoids in *fleur de sel* was related to the concentration of the microalgae *Dunaliella salina* (Donadio *et al.*, 2011), and the detection of plasticizers and fragrances in different samples of sea salts was related to the anthropogenic activity (Serrano *et al.*, 2011).

Among the volatile compounds identified in sea salt, the norisoprenoid β-ionone (a carotenoid-derived aroma compound) (**Fig. 1.10**), which exhibits a violet odour descriptor, was considered a potential contributor to the sea salt aroma (Silva *et al.*, 2010b).

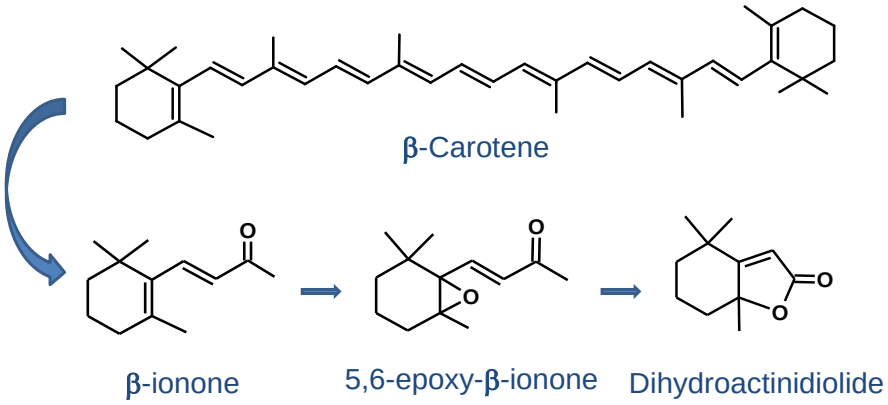

Figure 1.10. Example of a degradation pathway for β-carotene.

β-Ionone was quantified in sea salt samples from different geographical origins (Portugal, France, and Cape Verde), ranging from 241 to 888 ng/kg of salt (Silva *et al.*, 2010). Other carotenoid-derived aroma compounds, such as α-ionone and β-ionone-5,6-epoxide, were previously identified in sea salt (Silva *et al.*, 2009). Carotenoids are significant potential sources of volatile compounds in nature. These are polyene hydrocarbons biosynthesized from eight isoprene units (tetraterpenes) and their biosynthesis only occurs in bacteria, fungi, algae and plants (Belitz *et al.*, 2004; Rodriguez-Bustamante & Sanchez, 2007). Carotenoids are unstable lipophilic unsaturated structures, and their double bonds are highly susceptible to non-enzymatic (photo-oxygenation, auto-oxidation, thermal degradation) and enzymatic degradation (co-oxidation). The volatile carotenoid derivatives resulting from these degradation processes are called norisoprenoids (Rodriguez-Bustamante & Sanchez, 2007) (**Fig. 1.11**).

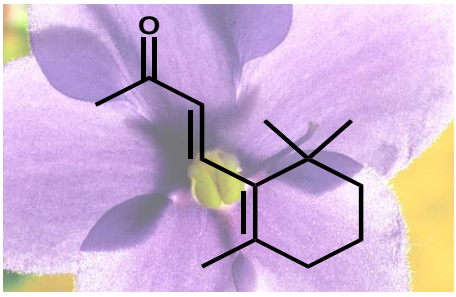

Figure 1.11. Chemical structure of β-ionone.

In spite of the useful data extracted from GC–qMS, the complexity of the volatile composition of sea salt exceeds the capacity of one single separation process, and the observed chromatographic co-elution limited reliable MS identification. This suggests that if a more powerful separation technique is used, a better characterisation of the sea salt volatile compounds can be obtained. In addition, the presence in sea salt of compounds in trace amounts also demands the use of a more sensitive technique. Comprehensive two-dimensional gas chromatography (GC×GC) should represent a suitable powerful tool for this study.

In recent years considerable research has been dedicated to the combination of independent techniques with the aim of strengthening resolving power (Kidwell *et al.*, 2004; Tranchida *et al.*, 2004). GC×GC combined with time-of-flight mass spectrometry (ToFMS) detection represents a successful example of this combination. GC×GC employs two orthogonal mechanisms to separate the constituents of the sample within a single analysis, based on the application of two GC columns coated with different stationary phases. Thus, a non-polar / polar phase combination, connected in series through a modulator interface, achieves this goal (**Fig. 1.12**).

The modulator has three main functions: (i) accumulate and trap, (ii) refocus, and (iii) rapidly release the adjacent fractions of the ^{1}D column (Adahchour *et al.*, 2008). The interface samples small (several seconds) portions of the first dimension (^{1}D) eluate by cryofocusing, and re-injects them into the second column (^{2}D). Each ^{1}D peak is modulated several times, largely preserving the ^{1}D separation. The second column is very short and narrow and, consequently, each modulated portion is “flash” separated before the next modulation. There are several types of jet-base modulators with either carbon dioxide or

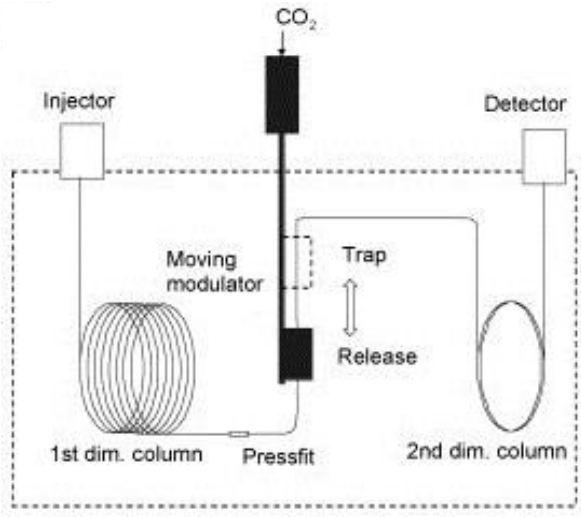

Figure 1.12. Schematic design of a comprehensive two-dimensional gas chromatograph including LCMS modulator. (Addapted from Adahchour *et al.*, 2008)

(liquid) nitrogen for cooling. **Fig. 1.12** shows a longitudinally modulated cryogenic system (LMCS), used in this PhD thesis. This modulator involves four steps, as illustrated in **Fig. 1.13**: (i) entrance of the broad peak into the trap, (ii) entrapment and focusing, (iii) moving the trap toward the injector, (iv) heating and remobilization (Marriott & Kinghorn, 1997).

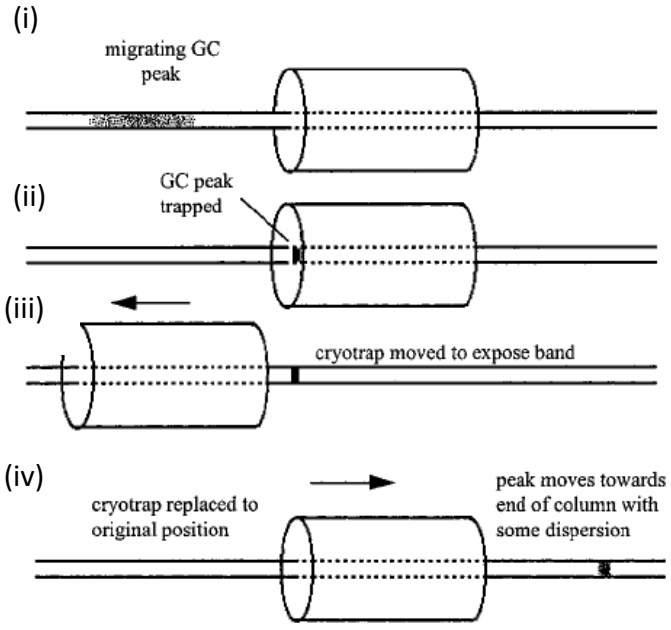

Figure 1.13. Schematic design of a LMCS modulation process. (Marriott et al., 1997)

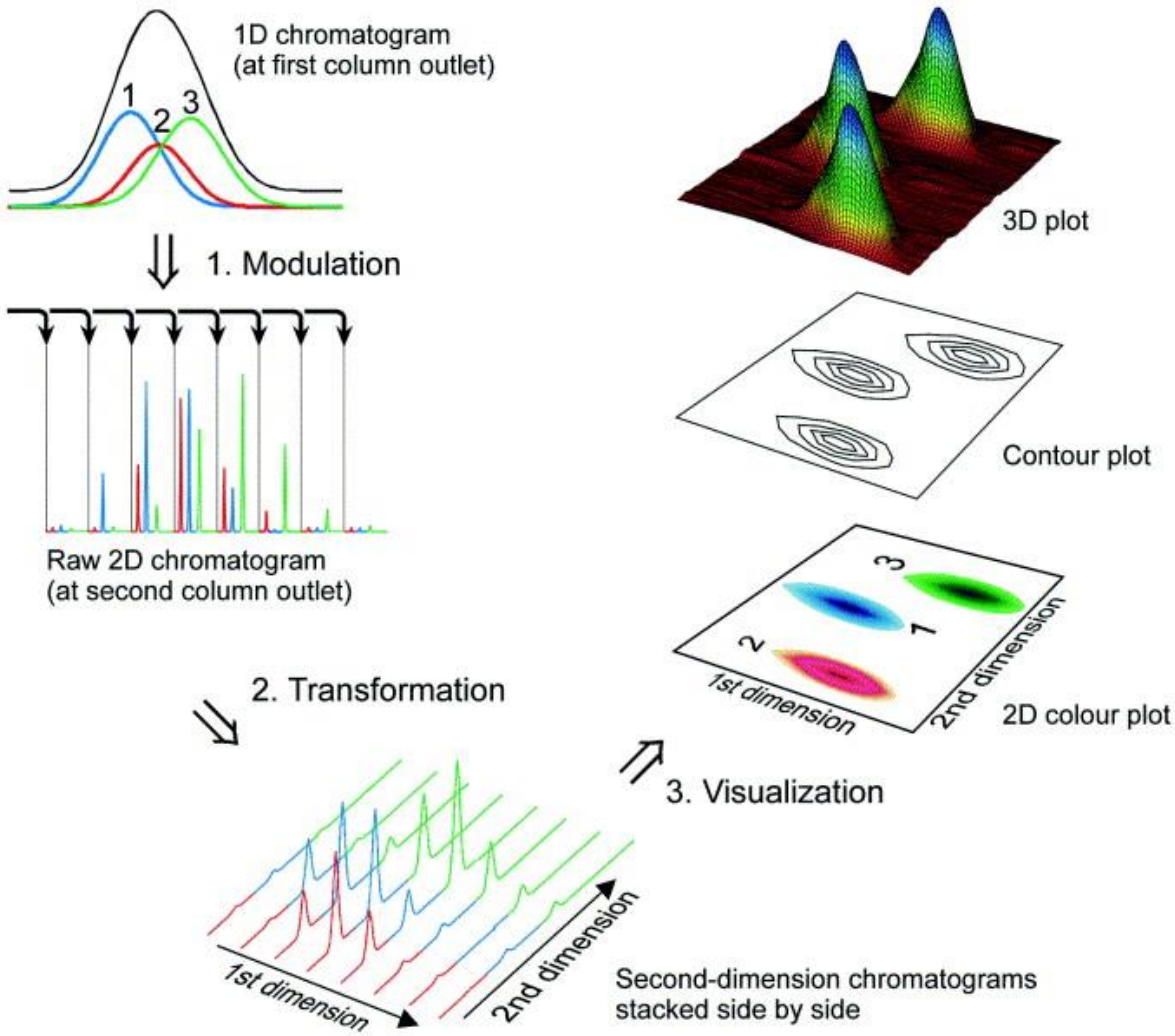

Figure 1.14. Generation and visualisation of a GC×GC chromatogram. (Dallüge *et al.*, 2003)

Using this instrumental approach, compounds co-eluting from ^{1}D undergo additional separation on ^{2}D (Górecki *et al.*, 2006). The process for the generation and visualisation of a GC×GC chromatogram, are shown in **Fig. 1.14** (Dallüge *et al.*, 2003). This shows the first step of modulation, in which the 1D chromatogram is sliced in a large series of high-speed 2D chromatograms, the second step of transformation, where these are placed side by side to form a 2D chromatogram, and the visualisation step where peaks are displayed in a 2D plane by means of colour, shading or contour lines to indicate the signal intensity (Dallüge *et al.*, 2003). Therefore, separation potential is greatly enhanced when compared to one-dimensional GC (1D-GC). Sensitivity and limits of detection (LoD) are

improved due to focusing of the peak in the modulator and separation of analytes from chemical background (Zrostlíková *et al.*, 2003). Signal-to-noise ratio is enhanced for GC×GC, compared to 1D-GC (Dallüge *et al.*, 2003; Marriott *et al.*, 2002).

Narrow peaks with width at half height of 0.1 s or less are preferably recorded by using high data acquisition speed of ToFMS to provide sufficient data density required for GC×GC separations (Shellie *et al.*, 2001). ToFMS brings other advantages such as full mass spectra acquisition at trace level sensitivity and mass spectral continuity, which allows for deconvolution of spectra of co-eluted peaks.

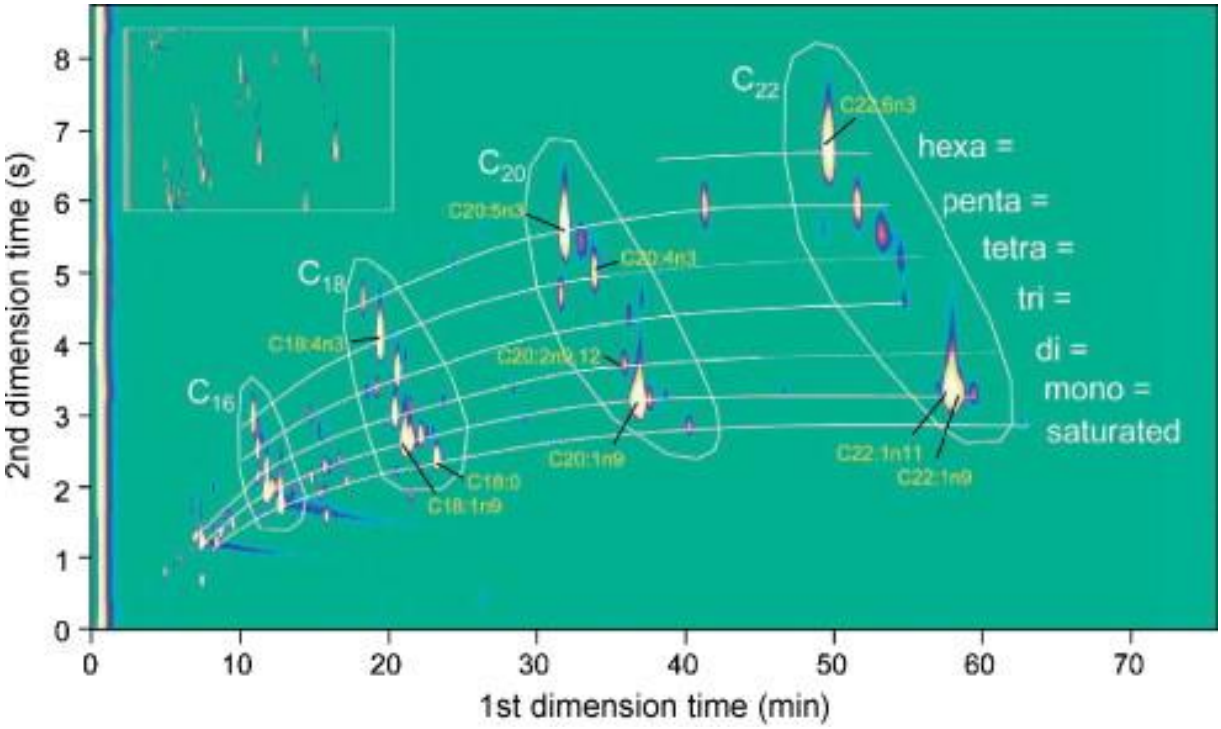

Figure 1.15. Example of a structured GC×GC chromatogram of fatty acids with different number of unsaturations and carbon chains (C_{16} – C_{22}). (Adahchour *et al.*, 2008)

Regarding data analysis, the software developed for GC×GC–ToFMS (ChromaTOF^{TM}) employs advanced True Signal Deconvolution$^{®}$ and automated peak find algorithms, comparison, and classification functions. All these features represent advantages in the data processing and analysis. Another advantage, allowing more reliable identifications, is the structured GC×GC (2D) chromatogram. When, the GC×GC analysis

was performed on a system comprising a non-polar (nP) ^{1}D column and a ^{2}D column polar (P) stationary phase, two almost independent separations (orthogonality) was observed. On the nP column, analytes were separated according to their vapour pressure/volatility, and on the P column, analytes were separated according to their polarity. Thus, chemically-related compounds have related spatial distributions in 2D chromatogram, as illustrated in **Fig. 1.15**.

GC×GC–ToFMS has been successfully used in several fields of analysis, including food and environment (Cardeal *et al.*, 2008; Humston *et al.*, 2009; Perestrelo *et al.*, 2011; Petronilho *et al.*, 2013; Rocha *et al.*, 2007; Rocha *et al.*, 2012; Tran *et al.*, 2007; Tranchida *et al.*, 2004). This methodology has never been reported before to study the volatile components of sea salt.

1.3.2. *Non-volatile fraction*

No references were found about the presence of non-volatile organic matter in sea salt. However, as volatile compounds are retained during sea salt crystallization, other organic compounds arising from the surrounding environment of saltpans could also remain associated to this natural product. In view of the possible sources described above, the present study focused on the occurrence of polymeric material, including polysaccharides and proteins, as well as in the presence of triacylglycerides. These represent important biomolecules that constitute, and are also produced, by the majority of organisms.

Polysaccharides

Polysaccharides containing neutral sugars (e.g. glucose, galactose, mannose, rhamnose, arabinose, xylose, ribose, and fucose), amino sugars (e.g. galactosamine and glucosamine) (Kaiser & Benner, 2000), and acidic sugars (Mopper, 1977; Leppard, 1995), mainly uronic acids (e.g. glucuronic acid and galacturonic acid), phosphorylated and sulfated sugars (Kaiser & Benner, 2000) have already been found in seawater (Engel & Händel, 2011). Present in marine organisms such as phytoplankton (Englel *et al.*, 2011), algae (Innamorati, 1995), and bacteria (Al-Nahas *et al.*, 2011), as structural components

and for energy storage (Engel & Händel, 2011), polysaccharides could be released into the medium by cell lysis, apoptosis or exudation (Innamorati, 1995; Giani *et al.*, 2005; Urbani *et al.*, 2005; Engel & Händel, 2011). These may end, for example, as mucilaginous aggregates drifting in seawater (complex mixture of organic matter) (Giani *et al.*, 2005; Cappiello *et al.*, 2007; Sartoni *et al.*, 2008). Besides seawater that supplies saltpans, polysaccharides are also found in microorganisms colonizing these same saltpans. Previous studies reported the production of polysaccharides by halophilic microalgae (Mishra *et al.*, 2011) and bacterial species (Philippis *et al.*, 1993; Cojoc *et al.*, 2009). Glucose, galactose, fructose, and xylose were identified as constituents of polysaccharides from microalga *Dunaliella salina* (Mishra *et al.*, 2011), glucuronic acid, galacturonic acid, galactose, glucose, mannose, xylose, and fucose, as constituents of polysaccharides from cyanobacterium (Philippis *et al.*, 1993), and polysaccharides containing amine and sulfate groups, found in halophilic bacteria (Cojoc *et al.*, 2009).

An important group of polysaccharides that may be present in seawater, and that comes mainly from macroalgae, are sulfated polysaccharides (Mollet *et al.*, 1998; Jiao *et al.*, 2011). These are also abundant in marine invertebrates (Bondu *et al.*, 2010; Mestechkina & Shcherbukhin, 2010). The major sulfated polysaccharides synthesised by algae are galactans (e.g. agarans and carrageenans), associated to red algae, ulvans, associated to green algae, and fucoidans, associated to brown algae (Jiao *et al.*, 2011). For instance, there are at least 15 different carrageenan structures, including κ, ι, and λ-carrageenan (**Fig. 1.16a**) (Jiao *et al.*, 2011). The main repeating disaccharide structures of ulvan contain sulfated rhamnose linked to either glucuronic acid or iduronic acid (**Fig. 1.16b**) (Jiao *et al.*, 2011). Fucoidans, well studied sulfated fucans, have mostly complex chemical compositions, although for some algae, such as *Fucus vesiculosus*, they exhibit a simple structure (**Fig. 1.16c**) (Li *et al.*, 2008).

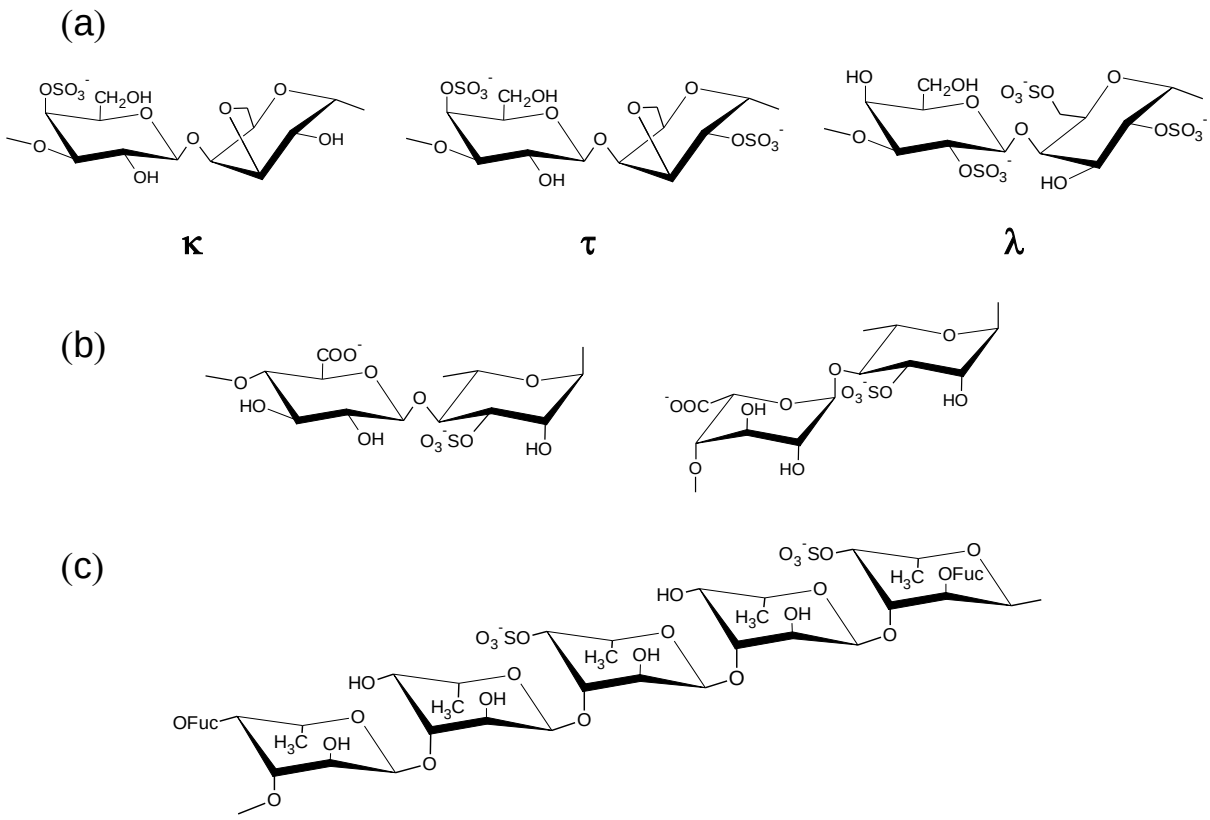

Figure 1.16. Examples of repeating structures of sulfated polysaccharides. (a) κ, ι, and λ-carrageenans (Jiao *et al.*, 2011), (b) ulvan (Jiao *et al.*, 2011), and (c) fucoidan (Li *et al.*, 2008).

High molecular weight material from algae was already obtained by means of extraction with cold water (Murano *et al.*, 1997). In this study, water soluble polysaccharides were then isolated after treatment with amylase, alcohol precipitation, dialysis and freeze drying (Murano *et al.*, 1997). A dialysis-based methodology seems to be suitable to extract polymeric material from sea salt.

Proteins

Such as polysaccharides, proteins are present in seawater (Lee *et al.*, 2000; Dittmar *et al.*, 2001; Sommerville & Preston, 2001; Tsukasaki & Tanoue, 2010), where they constitute 20-80% of the dissolved organic nitrogen (Sommerville & Preston, 2001). Representing also the greater reservoir of organic nitrogen of most organisms (Lee *et al.*, 2000), they can be released into the media by marine organisms, such as phytoplankton

(Sommerville & Preston, 2001; Metaxatos *et al.*, 2003), cyanobacteria (Giani *et al.*, 2005), bacteria (Pistocchi *et al.*, 2005), and algae (Leppard, 1995). As for polysaccharides, these may be released into the medium by means of cell lysis, apoptosis or exudation. Regarding amino acids composition, alanine, glycine, valine, threonine, serine, leucine, isoleucine, proline, aspartic acid, phenylalanine, glutamic acid, methionine, arginine, histidine, tyrosine, and lysine, coming from protein, have already been identified in seawater (Lee *et al.*, 2000; Dittmar *et al.*, 2001; Sommerville & Preston, 2001; Tsukasaki & Tanoue, 2010). For example, arginine and glutamic acid were previously identified as predominant in mucilage material of phytoplankton (Metaxatos *et al.*, 2003), while aspartic and glutamic acids are among the major amino acids in algae (Dawczynski *et al.*, 2007; Kumar & Kaladharan, 2007; Gressler *et al.*, 2011) and phytoplankton (Granum *et al.*, 2002). Proteins are also found in microorganisms living in hyper saline habitats, such as microalgae and bacterial species (Mishra *et al.*, 2008; Bardavid & Oren, 2012; Yildiz *et al.*, 2012). For instance, amino acids such as glutamic acid, aspartic acid, lysine, arginine, glutamine, and asparagine were previously identified in proteins of halophilic bacteria (Bardavid & Oren, 2012).

Triacylglycerides

Triacylglycerides (TG) are esters derived from glycerol and three fatty acids, and represent part of a larger group of biomolecules known as lipids. These are neutral lipids. Lipids are structural components and a form of energy storage of plants and animals (Napolitano *et al.*, 1997), and so, widely present in the marine environment. Composed by saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated fatty acids (PUFAs), triacylglycerides are known constituents of the marine biota, including algae (Gressler *et al.*, 2011; van Ginneken *et al.*, 2011; Guedes *et al.*, 2011), phytoplankton (Napolitano *et al.*, 1997; Chen, 2012), and cyanobacteria (Guedes *et al.*, 2011), and could be released into the medium by the same way as the polysaccharides and proteins. Despite the numerous possible natural sources, the presence of lipids in seawater can also derive from contaminations, consequence of anthropogenic activities, such as urban wastes (Marty *et al.*, 1996). TG have already been found as the major lipid class of marine organisms such as phytoplankton (Henderson & Mackinlay, 1989), and microalgae (Alonso *et al.*, 1998).

There are several studies on fatty acid composition of lipids extracted from marine organisms, whereas studies focusing in particular triacylglycerides are few. For instance, large amounts of palmitic acid (C16:0) (Gressler *et al.*, 2011; van Ginneken *et al.*, 2011), oleic acid (C18:1), arachidonic acid (C20:4), and eicosapentaenoic acid (C20:5) (van Ginneken *et al.*, 2011) were already identified in macroalgae. PUFAs are often found in great majority (Dawczynski *et al.*, 2007; van Ginneken *et al.*, 2011). High proportions of palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), and eicosapentaenoic (C20:5(n-3)) fatty acids, were previously identified in TG from marine microalgae (Alonso *et al.*, 1998). Palmitic acid (C16:0), linoleic acid (C18:2), and α-linolenic acid (C18:3) are among the main fatty acids of cyanobacteria (Guedes *et al.*, 2011). A study on fatty acids in marine atmosphere, obtained from lipid analysis of aerosol samples, showed a positive correlation of the amount of lower molecular weight (C_{14}-C_{19}) SFAs, likely originated from marine biota, with sea salt concentration, suggesting that these are released from the ocean surface to the atmosphere with sea salt particles. Palmitic, myristic (C14:0), and stearic acids were the three major SFAs identified (Mochida *et al.*, 2002). As a result of anthropogenic activity, C16:0 along with C18:0 were already identified in seawater, being the main components of the triacylglycerides present due to urban wastes (Marty *et al.*, 1996).

1.4. MAIN GOALS

The growing concern for the protection and revalorisation of saltpans identity and survival of the natural and cultural heritage associated to them is intrinsically associated to the quality of the sea salt. This can be evaluated by its physico-chemical proprieties, representing a step towards a deeper differentiation of this natural product. Based on the hypothesis that sea salt contains organic compounds associated to their crystals that can be used as markers of the sea salt itself, including saltpans surrounding environment, the aim of this PhD thesis was to identify these compounds. With this purpose, three main goals were established for the present work (**Fig. 1.17**):

- To obtain a deep characterisation of the volatile composition of sea salt by means of HS-SPME combined with comprehensive two-dimensional gas chromatography

time-of-flight mass spectrometry (GC×GC–ToFMS) methodology, in search of potential volatile markers;

- To develop a methodology to isolate the polymeric material potentially present in sea salt, in amounts that allow its characterisation in terms of polysaccharides and protein;

- To explore the possible presence of triacylglycerides in sea salt.

The achievement of these goals will allow to evaluate the possibility of a typical pattern regarding the organic matter associated to sea salt, and the presence of potential molecular markers of this product.

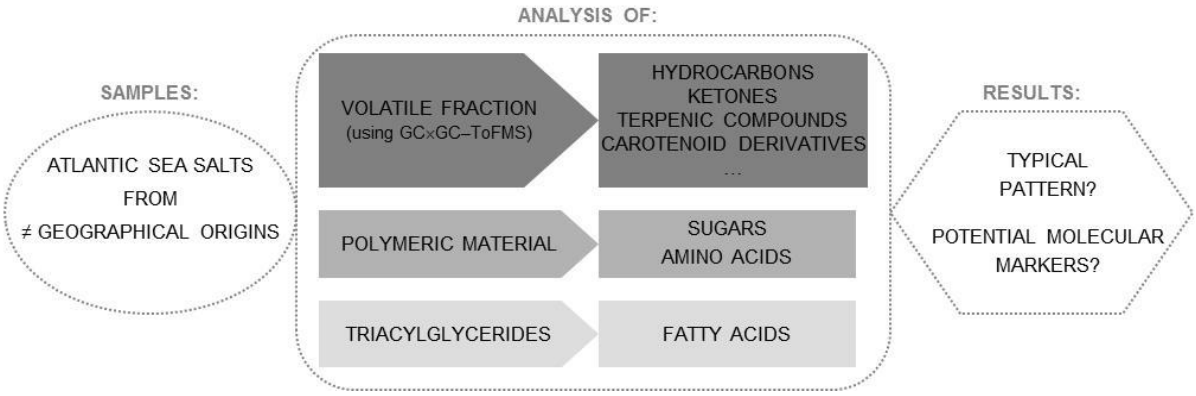

Figure 1.17. Main goals scheme of the present PhD thesis.

2.1. SAMPLES

Sea salt samples from several geographical origins were analysed in this study (**Fig. 2.1**). Although all saltpans are located in the Atlantic Ocean, according to their geographical origin they can be influenced by different Ocean currents (**Fig. 2.1**). Sea salts coming from 9 locations and produced from 2004 to 2009 were analysed (**Table 2.1**).

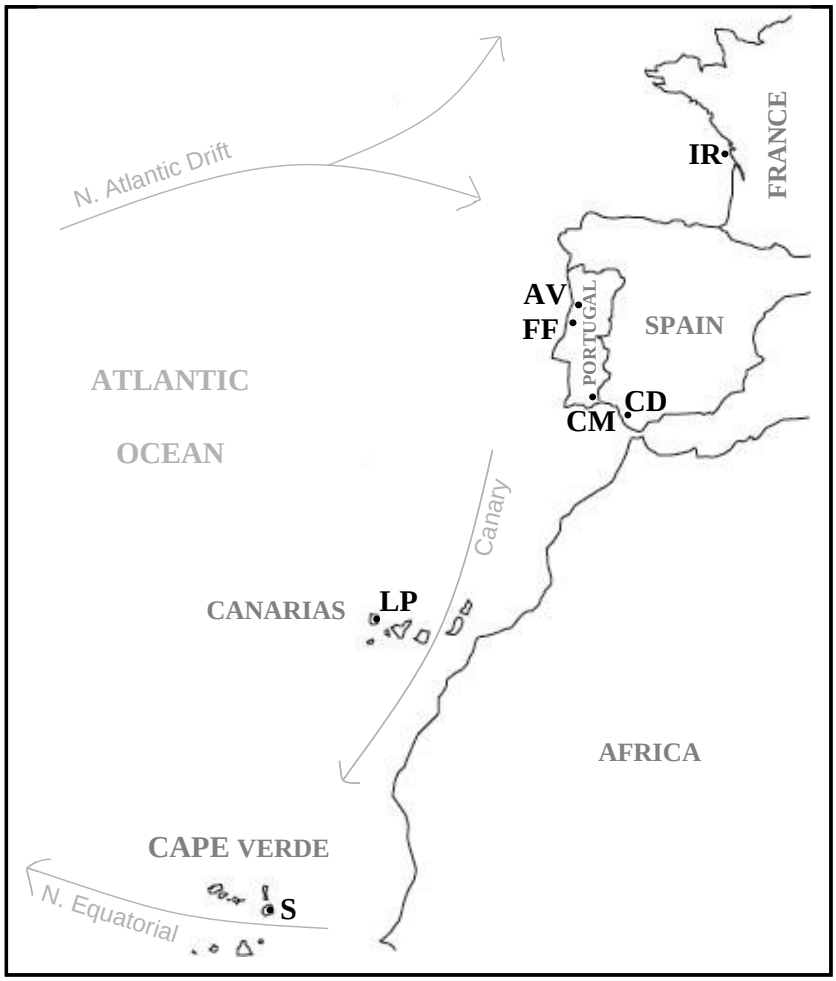

Figure 2.1. Map showing the sea salt sampling sites in the Atlantic Ocean. *Île de Ré* – **IR**; *Aveiro* - **AV**; *Figueira da Foz* – **FF**; *Castro Marim* – **CM**; *Cádiz* – **CD**; *La Palma island* – **LP**; *Sal island* – **S**. The Atlantic Ocean currents are also highlighted: North Atlantic Drift, Canary, North Equatorial.

Table 2.1. Sea salt samples analysed in the present study.

ORIGIN	YEAR OF PRODUCTION	ANALYSIS PERFORMED [1] (YEAR)
AVEIRO (**AV**) **PJ** **18C** **GCa**	 2004/ 2005/ 2007 2004/ 2005/ 2007 2007	 Volatiles; (2008) Sugar; (2011) Sulfate; (2011) Protein; (2010) Triacylglycerides (2011)
FIGUEIRA DA FOZ (**FF**)	2004 2005 2007	Sugar; Sulfate; Protein; Triacylglycerides Sugar; Sulfate; Protein; Triacylglycerides Volatiles; Sugar; Sulfate; Protein; Triacylglycerides
CASTRO MARIM (**CM**)	2007	Volatiles; Sugar; Sulfate; Protein; Triacylglycerides
CÁDIZ (**CD**)	2007	Volatiles; Sugar; Sulfate; Protein; Triacylglycerides
ÎLE DE RÉ (**IR**)	2007	Volatiles; Sugar; Sulfate; Protein; Triacylglycerides
LA PALMA (**LP**)	2007 2009	Volatiles; Sugar; Sulfate; Protein; Triacylglycerides Sugar; Sulfate; Protein; Triacylglycerides
SAL (**S**)	2007	Volatiles; Sugar; Sulfate; Protein; Triacylglycerides

[1] Each analysis was performed at the same time for all sea salts under study

Aveiro (AV) saltpans are located in the north coast of Portugal, more exactly in Vouga river lagoon (Ria de Aveiro), at 8 km from the sea (**Fig. 2.2**). The samples of sea salt from Aveiro came from three different saltpans, namely *Peijota* (PJ), *18 dos Caramonetes* (18C) and *Grã Caravela* (AV or GCa).

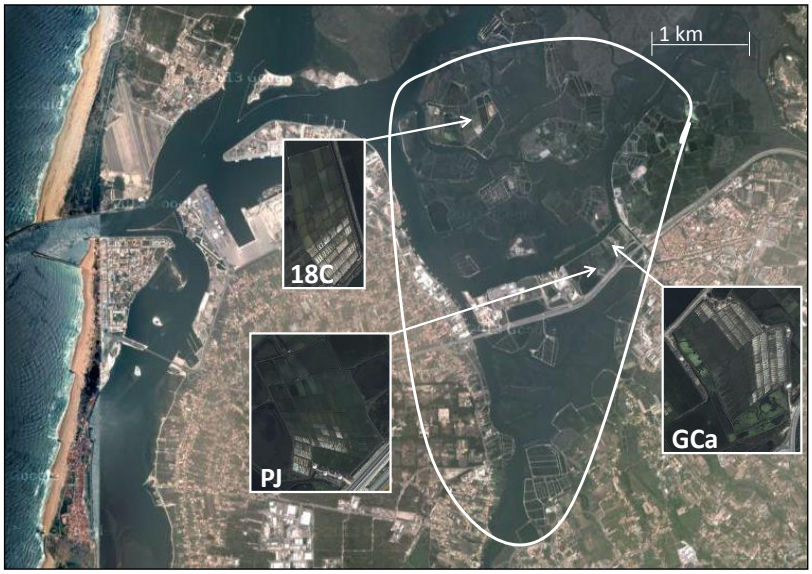

Figure 2.2. Satellite image showing Aveiro saltpans location. *Peijota* – **PJ**; *18 dos Caramonetes* – **18C**; *Grã Caravela* – **GCa**.

(Satellite image obtained from Google maps)

Also from the north coast of Portugal, in the *Mondego* river margins, is located the Figueira da Foz (FF) saltpan, at 3 km from the sea, more precisely in *Murraceira* island (**Fig. 2.3**).

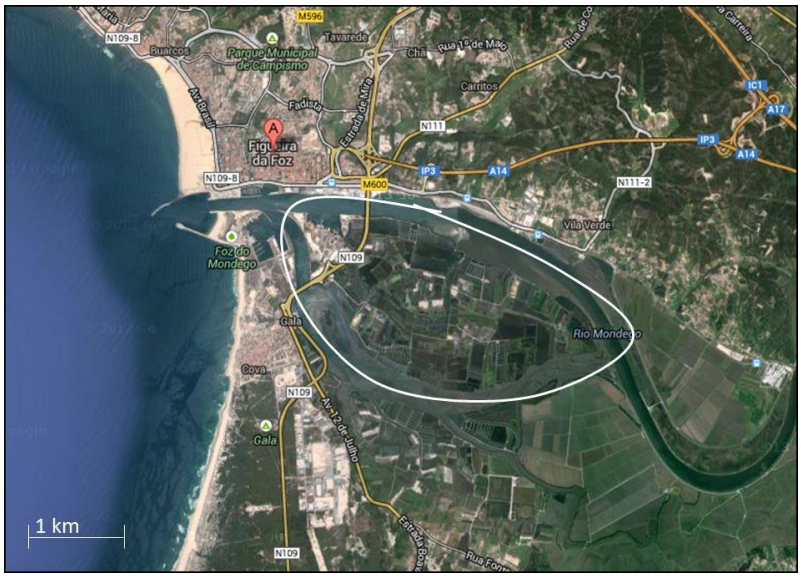

Figure. 2.3. Satellite image showing Figueira da Foz saltpans location.

(Satellite image obtained from Google maps)

In Guadiana river margins, Algarve, south of Portugal, at 5 km from the sea, and with Mediterranean sea influence, is located the Castro Marim (CM) saltpan (**Fig. 2.4**).

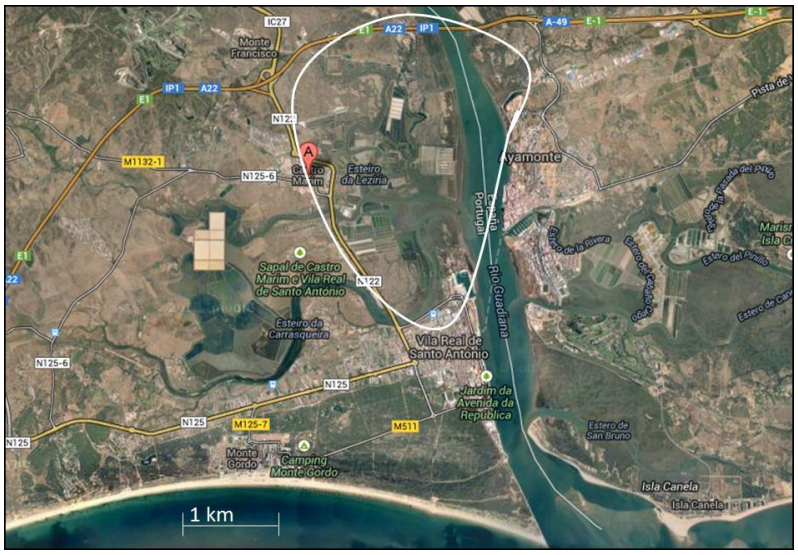

Figure 2.4. Satellite image showing Castro Marim saltpans location.

(Satellite image obtained from Google maps)

Also with Mediterranean influence, island of *Léon*, in Andalucia, Southwestern Spain, comprises the saltpan of Cádiz (CD), located in the margins of *Zurraque* river, at 7 km from the sea (**Fig. 2.5**).

Figure 2.5. Satellite image showing Cádiz saltpans location.

(Satellite image obtained from Google maps)

Île de Ré (IR) saltpan is located at 1 km from the sea (**Fig. 2.6**), in the Western Cost of France.

Figure 2.6. Satellite image showing Île de Ré saltpans location.

(Satellite image obtained from Google maps)

In Canarias Islands, La Palma (LP) (**Fig. 2.7**) saltpan is located at 2 km from the sea, in an arid zone.

Figure 2.7. Satellite image showing La Palma island saltpans location.

(Satellite image obtained from Google maps)

Sal island (S) (**Fig. 2.8**) saltpan, in Cape Verde, is an inland saltpan located at 2 km from the sea, in an arid zone. Due to the proximity of the sea, the salt produced in the inland saltpan of Sal island is considered sea salt.

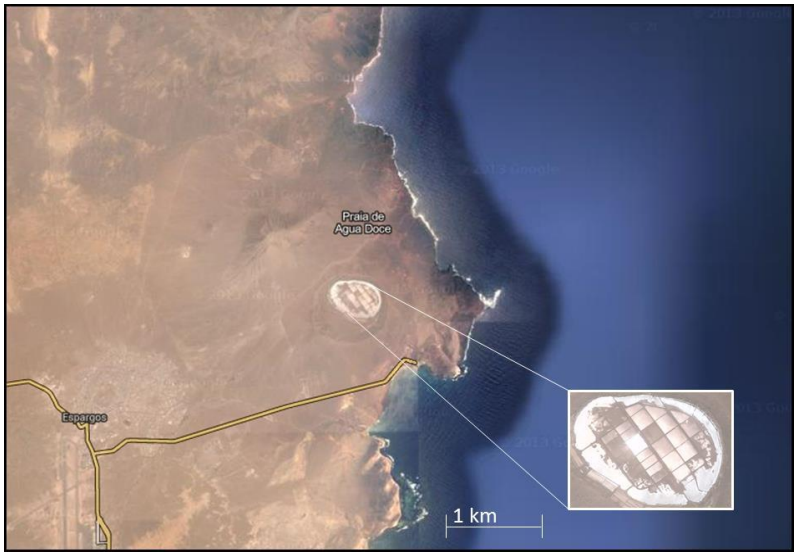

Figure 2.8. Satellite image showing Sal island saltpans location.

(Satellite image obtained from Google maps)

All the sea salt samples, except the one from Cape Verde, obtained from a local store, were supplied by the participants of the project SAL – Sal do Atlântico.

For a comparative study, two salt samples from an inland origin, far from the sea, were also analysed in terms of their volatile composition. These samples were collected in aquifers saline of Murray Darling Basin, in Australia, from Mildura, in the North-western of the state of Victoria, a region situated more than 200 km from the sea (**Fig. 2.9**). This inland salt is not considered sea salt. These samples, identified as Coarse Gold (CG) and Pink Flakes (PF), were produced in 2008 and supplied by the Australian trading company SunSalt.

Figure 2.9. Satellite image showing Australia saltpans location.

(Satellite image obtained from Google maps)

All samples under study were stored in glass bottles until analysis.

2.2. HS-SPME METHODOLOGY

Both the SPME holder for manual sampling and the fibre were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a flexible fused silica core bonded with a mixed phase coating of 50/30 µm divinylbenzene/carboxen on polydimethylsiloxane (DVB/CAR/PDMS). This coating combines the absorption properties of the non-polar liquid polymer PDMS with the adsorption properties of the

porous particles of divinylbenzene solid polymer and carbon molecular sieve (CAR) containing macro- (>500 Å), meso- (20–500 Å) and micro-pores (2–20 Å). This fibre is recommended for the analysis of volatile and semi-volatile compounds (C_3–C_{20}) within a molecular weight range from 40 to 275. The SPME fibre was conditioned at 270ºC for 60 min in the GC injector, according to the manufacturer's recommendations.

The HS-SPME methodology used in this study was based on the previous work developed by Silva *et al.* (2009), although three parameters have been modified: type of SPME coated fibre, mode of sample presentation (solid crystals instead of aqueous saturated salt solution), and extraction time. As the 65 µm carbowax/divinylbenzene (CW/DVB) SPME fibre previously used (Silva *et al.*, 2009) is no longer commercially available, a StableFlex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating that also presents a wide range capacity of sorbing compounds with different physico-chemical properties, was used. Taking into account the higher sensitivity of GC×GC–ToFMS compared to GC–qMS, the sea salt was analysed as solid crystals, reducing the time of sample preparation and avoiding any possible artefacts resulting from the added water. The SPME extraction time was reduced from 90 to 60 min. Briefly, ~ 6 g ($1/\beta$=0.5) of sea salt were added to a 22 mL vial. The vial was capped with a PTFE septum and an aluminium cap (Chromacol Ltd., Herts, UK), and to reach the equilibrium it was placed in a dry heat block adjusted to 60.0 ± 0.1ºC overnight. The SPME fibre was then manually inserted into the sample vial headspace for 60 min. In order to avoid any cross-over contamination due to own fibre, blanks, corresponding to analysis of the coated fibre not submitted to any extraction procedure, were run between sets of three analyses. Three independent aliquots of each sea salt were analysed. Reproducibility was expressed as relative standard deviation (RSD).

2.3. GC×GC–ToFMS ANALYSIS

The SPME coated fibre with sorbed sea salt volatile compounds was manually introduced into the GC×GC injection port at 240ºC and kept for 5 min for desorption. Splitless injections were used (5 min). The desorbed volatile compounds were separated in a GC×GC–ToFMS system (**Fig. 2.10**) comprising of a HP 6890 (Agilent Technologies,

Burwood, Australia) gas chromatograph and a Pegasus III time-of-flight mass spectrometer (LECO, St. Joseph, MI, USA). To implement the modulation process, a longitudinally modulated cryogenic system (LMCS; Chromatography Concepts, Doncaster, Australia) was used, operated at a modulation period of 5 s with a cryotrap temperature of −20ºC. This modulation period maximized the 2D resolution, and avoided the wrap-around effect for most polar compounds. Ideally, all peaks must be detected before the subsequent reinjection, and hence, $^{2}t_{R}$ (retention time in the second dimension, ^{2}D) must be equal or less than the modulation period (Dallüge *et al.*, 2003; Mondello *et al.*, 2008). The ToFMS was operated at a storage rate of 100 Hz, using a mass range of *m/z* 41–415 and a multi-channel plate voltage of 1600 V. The column set used for GC×GC experiments comprised a BPX5 (5% phenyl polysilphenylenesiloxane phase) primary column; 30 m × 0.25 mm I.D., 0.25 μm film thickness (d_f), directly coupled to a BP20 (polyethylene glycol phase) second column of 1.0 m × 0.1 mm I.D., 0.1 μm d_f (both columns from SGE International, Ringwood, Australia). The GC oven temperature program was: initial temperature 50ºC (hold 3 min), raised to 130ºC (10ºC min^{-1}), then raised to 230ºC (5ºC min^{-1}) (hold 5 min). Helium was used at a flow rate at 1.0 mL min^{-1}. The transfer line for the GC×GC–ToFMS system was a 0.50 m deactivated fused silica column of 0.1 mm I.D. (0.21 m inside the interface and 0.29 m inside the oven) from SGE International. Data were processed using LECO Corp. ChromaTOF^{TM} software. Contour plots were used to evaluate the general quality of the separation and for manual peak identification. A signal-to-noise threshold of 100 was used. Tentative identification of compounds was achieved by comparing the experimental mass spectra with database libraries (Wiley 275 and National Institute of Science and Technology Mass Spectra Library (NIST 2.0, 2005) - Mainlib and Replib), and supported by experimentally determined retention index (RI) values that were compared, when available, with values reported in the bibliography for chromatographic columns, equivalent to that used as the ^{1}D column in the present work (Georgilopoulos & Gallois, 1987; Adams, 1995; Elmore *et al.*, 1999; Högnadóttir & Rouseff, 2003; Valim *et al.*, 2003; Leffingwell & Alford, 2005; Pino, 2007; Zeng *et al.*, 2007; Hoet *et al.*, 2010; Babushok & Zenkevich, 2009; M^{c}Ginitie & Harynuk, 2012; Vasta *et al.*, 2012).

Figure 2.10. Photo of a HP 6890-Pegasus III GC×GC–ToFMS instrument.

For determination of RI values a C_8–C_{20} *n*-alkanes series was used. These were calculated according to the Van den Dool and Kratz equation (Van den Dool & Kratz, 1963). The majority (>80%) of the identified compounds presented similarity matches >800. The DTIC (Deconvoluted Total Ion Current) GC×GC area data were used as an approach to estimate the relative content of each volatile component.

2.4. SEA SALT MOISTURE CONTENT

The moisture content of the salts under study was determined in order to express the content of polymeric material (PM) as sea salt dry weight. To determine the moisture content of the sea salts under study, 5 g of each sample were dried in an oven at 110ºC during 12 h (Koloff *et al.*, 1969; Banca *et al.*, 2002). The weight of each sea salt was recorded after cooling to room temperature, in a desiccator. Three independent aliquots of each sample were analysed.

2.5. ISOLATION OF POLYMERIC MATERIAL FROM SEA SALT

For the isolation of the PM from sea salt crystals in quantitative amounts in order to allow its analysis, a methodology needed to be developed (**Fig. 2.11**). First, dialysis membranes (size 5, 12–14 kDa, Medicell), with a volume of approximately 80 mL, were filled directly with 60 g of sea salt crystals. This procedure was carefully preformed not to damage the membrane due to the crystals sharp edges. Then, distilled water was added to fill the remaining air gaps and the dialysis membranes were sealed avoiding the presence of air inside. For each sea salt, 8 membranes were prepared. The filled membranes were placed in one jar containing 10 L of distilled water. The dialysis occurred during approximately one week, under stirring, with 2-3 times daily water exchanges. Five drops of toluene and five drops of chloroform were added always to the new dialysis water in order to avoid microbial contamination. The dialyses ended when the conductivity of the dialysate water after 6 h was similar to that of the distilled water. Then, the content of the dialysis membranes were pooled and concentrated by rotary evaporation at 30ºC until a volume of 30 mL. To eliminate the presence of any sea salt, the concentrated solution was again dialysed in 10 L of water for more two days with 2-3 daily water exchanges, controlling the conductivity of the dialysate. The retentate was then frozen and freeze-dried to obtain the PM from sea salt. Due to its hygroscopicity, it was stored in a desiccator with P_2O_5 until analysis to avoid water absorption.

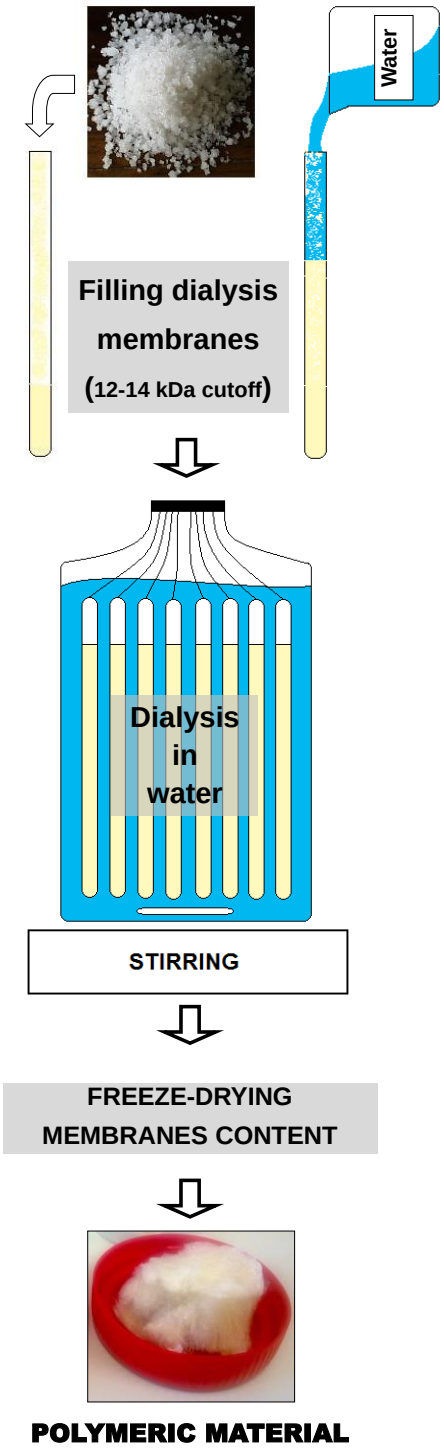

Figure 2.11. Dialysis-based methodology to isolate polymeric material from sea salt.

2.6. MID-INFRARED SPECTROSCOPY

The PM isolated from sea salt was characterised by mid-infrared spectroscopy in the 4000-500 cm^{-1} region, with 8 cm^{-1} resolution with 128 co-added scans, using a Golden-Gate single reflectance ATR in a Bruker IFS-55 instrument (Bruker, Karlsruhe, Germany). Five independent aliquots of each sample were analysed (Coimbra *et al.*, 1998).

2.7. THERMOGRAVIMETRIC ANALYSIS

A thermogravimetric analysis of PM from sea salt was also performed, using a Setaram analyser (SETSYS Evolution 1750) equipped with an alumina holder. PM (1–3 mg) were heated at a constant rate of 10ºC/min from room temperature to 600ºC under an oxygen flow of 200 mL/min. One aliquot of each sample was analysed.

2.8. SUGAR COMPOSITION AND LINKAGE ANALYSIS

Neutral sugars were analysed as their alditol acetates by GC-FID (Blakeney *et al.*, 1983; Harris *et al.*, 1988). PM from sea salt (1–2 mg) pre-hydrolysis was performed in 0.2 mL of 72% H_2SO_4 (w/w) for 3 h at room temperature, followed by 1 h hydrolysis in 2M H_2SO_4 at 120ºC. Neutral sugars were analysed by GC-FID after conversion to their alditol acetates, using 2-deoxyglucose as internal standard (Coimbra *et al.*, 1996). A Perkin-Elmer Clarus 400 gas chromatograph with split/splitless injector and a FID detector was used, equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA, USA) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. Samples were injected in split mode (5 min) with the injector operating at 220ºC. The GC oven temperature program was set at an initial temperature of 200ºC, raised to 220ºC at 40ºC/min, holding for 7 min, then raised to 230ºC at 20ºC/min, and held there for 1 min. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min. One aliquot of each sea salt sample of PM was analysed.

Uronic acids (UA) were determined by a modified version of the 3-phenylphenol colourimetric method of Blumenkrantz and Asboe-Hansen (1973) (Coimbra *et al.*, 1996).

The PM samples were submitted to a pre-hydrolysis with 0.2 mL of 72% H_2SO_4 (w/w) during 3 h at room temperature and under vigorous stirring. UA concentrations were estimated from a calibration curve build with D-galacturonic acid as standard.

Glycosidic-linkage analysis was carried out by methylation as described by Ciucanu and Kerek (1984) and Ciucanu (2006). Before analysis, the samples of PM from sea salt (3–4 mg) were placed overnight in a vacuum oven, at room temperature, in presence of P_2O_5. Then, the samples were dissolved in dimethyl sulfoxide (DMSO), powdered NaOH (40 mg) was added and samples were methylated with CH_3I (80 μL). After one hour reaction samples were dissolved in $CHCl_3$/MeOH (1:1, v/v) and dialysed against 50% EtOH. After dialysis samples were dried and submitted to a new methylation and subsequent dialysis. Part of the dried methylated fractions was carboxyl-reduced by a modification of the method described by Lindberg and Lönngren (1978) (Coimbra *et al.*, 1996), to determine which UA were present. The methylated fraction was heated in a sealed tube with a mixture of $LiAlD_4$ (20 mg) in tetrahydrofuran (THF) (1 mL) at 65ºC for 4h. The excess of reagent was then destroyed with ethanol (2-3 drops), and the pH of the mixture was adjusted to neutrality with 2 M H_3PO_4. The reduced polymer was isolated by filtration, washed thoroughly with $CHCl_3$: MeOH (2:1, v/v), evaporated to dryness. The methylated polysaccharides and the methylated and carboxyl-reduced polysaccharides were hydrolysed with 2 M trifluoroacetic acid (TFA) at 121ºC for 1 h and dried by centrifugal evaporation. The reduction of monosaccharides was performed during 1 h at 30ºC with 20 mg of $NaBD_4$ in 300 μL of 2M NH_3, and subsequently acetylated with acetic anhydride (3 mL) in presence of 1-methylimidazole (450 μL) for 30 min at 30ºC. Partially methylated alditol acetates (PMAA) were separated with CH_2Cl_2 and analysed by GC–qMS. An Agilent Technologies 6890N Network gas chromatograph with a split/splitless injector was used, equipped with a 25 m column DB-1 (QUADREX Corporation, New Haven, CT, USA) with i.d. and film thickness of 0.25 mm and 0.05 μm, respectively, and connected to an Agilent 5973 quadrupole mass selective detector. Samples were injected in splitless mode (5 min) with the injector operating at 220ºC. The GC oven temperature program was set at an initial temperature of 50ºC, raised to 140ºC at 8ºC/min, holding for 5 min, raised to 150ºC at 0.5ºC/min, then raised to 300ºC at 40ºC/min, holding for 1 min. The flow rate of the carrier gas (He) was set at 1.7 mL/min. The mass spectrometer was

operated in the electron impact mode (EI) at 70 eV scanning the range 40–500 *m/z*, in a full scan acquisition mode.

Chromatogram peaks were tentatively identified comparing all mass spectra with a laboratory made database of PMAAs. For those cases in which co-elution of PMAA residues was verified and, consequently, the estimation of the area corresponding to each compound was not possible, ion extraction chromatography (IEC) mode was used to estimate the relative proportion of the co-eluting compounds. Thus, abundant ions with exclusive *m/z* values were identified for each co-eluted compound, their chromatographic areas were obtained by IEC, and their relative contribution to the chromatographic area of the peak detected by total ion chromatography (TIC) was calculated.

2.9. DETERMINATION OF SULFATE

Sulfate content was determined by turbidimetry. This was carried out according to the turbidimetric method described by Dodgson and Price (1962) for determination of the ester sulfate content of sulfated polysaccharides.

First, 1-2 mg of PM from sea salt were dissolved in 1 M HCl (1 mL). A portion (0.5 mL) of the previous solution was hydrolysed at 110ºC for 5 h. After hydrolysis, a first aliquot (0.2 mL) was transferred to a suitable tube containing 3.8 mL of 3% (w/v) trichloroacetic acid and 1 mL of a barium chloride-gelatin reagent (0.5 g of $BaCl_2$ in 100 mL of gelatin solution). Gelatin (0.5 g in 100 mL of water) was used as a cloud-stabilizer of the barium sulfate formed. After reacting for 20 min, under stirring, the released barium sulfate suspension absorbance was measured at 360 nm against a reagent blank consisting of 0.2 mL of water, 3.8 mL of 3% (w/v) trichloroacetic acid and 1 mL of the barium chloride-gelatin reagent. A second aliquot (0.2 mL) of the hydrolysate was transferred to another tube containing 3.8 mL of 3% (w/v) trichloroacetic acid (TCA) and 1 mL of gelatin solution (i.e. containing no barium chloride) was added. The absorbance of this 'control' solution was then measured at 360 nm against a reagent blank consisting of 0.2 mL of hydrochloric acid, 3.8 mL of trichloroacetic acid and 1 mL of gelatin solution. This control represents the ultraviolet-absorbing compounds produced during hydrolysis. The resulting value was subtracted from that of the hydrolysate aliquot analysed in presence of

$BaCl_2$-gelatin reagent. Determination of sulfate was achieved with a calibration curve built with K_2SO_4 standard solutions (20 – 200 μg of SO_4^{2-} ions), with no less than two replicates per sample.

2.10. AMINO ACID COMPOSITION AND PROTEIN CONTENT

The freeze-dried PM from sea salt (2-5 mg) was submitted to acid hydrolysis with 6 M HCl, during 24 h at 110ºC (Zumwalt *et al.*, 1987), using 0.1 mL of 5 mM L-norleucine in 0.1 M HCl as internal standard (Coimbra *et al.,* 2011). The derivatization of amino acids to their *N*-heptafluorobutyryl isobutyl esters was preformed according to the methodology developed by Mackenzie & Tenaschuk (1974). The *N*-heptafluorobutyryl isobutyl esters of the amino acids were dissolved in 0.02 mL of ethyl acetate and analysed by GC–FID. A PerkinElmer Clarus 400 chromatograph (PerkinElmer, Massachusetts, USA) with split/splitless injector and a FID detector was used, equipped with a 30 m DB-1 (J & W Scientific) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. Samples were injected in split mode (5 min) with the injector operating at 250ºC. The GC oven temperature program was set at an initial temperature of 70ºC, holding for 1 min, raised to 170ºC at 2ºC/min, then raised to 250ºC at 16ºC/min, holding for 5 min. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min. The compounds were identified by their retention times and chromatographic comparison with authentic standards. Amino acids quantification was achieved with the internal standard method and external calibration curves previously built for 21 amino acids (Coimbra *et al.*, 2011). For asparagine (Asn) and aspartic acid (Asp), as well as for glutamine (Gln) and glutamic acid (Glu), the methodology does not allow the distinction between the amide and carboxylic acid functions. As such, those amino acids were quantified together as Asx and Glx, respectively. One aliquot of each sea salt sample of PM was analysed.

2.11. TRIACYLGLYCERIDES EXTRACTION AND FATTY ACID COMPOSITION

Sea salt (20 g) triacylglycerides were extracted with *n*-hexane (150 mL) in a Soxhlet apparatus (250 mL round bottom flask; Soxhlet chamber of 50 mL capacity; 23 mm × 100 mm cartridge) during 4 h (Passos *et al.,* 2009). The organic extracts of each salt were obtained from two independent aliquots. These were combined and evaporated under vacuum until all *n*-hexane was removed. The triacylglycerides obtained were then transesterified to determine fatty acids (FAs) composition by gas chromatography.

Fatty acid methyl esters (FAMEs) were prepared by transesterification with a methanolic KOH solution (Aued-Pimentel *et al.*, 2004; Passos *et al.*, 2010). As the amount of triacylglycerides extracted from the 2 × 20 g of sea salt was very small, the sample was directly dissolved in 0.5 mL of an internal standard solution of heptadecanoate methyl ester (C17:0) prepared in *n*-hexane (0.03 mg/ mL). Then, a methanolic KOH solution (2 M) was added (0.02 mL) and the resulting solution was mixed vigorously for 30 s in a vortex shaker. After, a saturated NaCl solution (0.2 mL) was added and the sample centrifuged at 2000 rpm during 5 min. The organic phase was transferred to a clean tube and evaporated under vacuum. FAMEs were dissolved in 0.1 mL of *n*-hexane and aliquots (0.2–0.5 µL) were used for GC–qMS analysis. An Agilent Technologies 6890N Network gas chromatograph with a split/splitless injector was used, equipped with a 25 m column DB-1 (QUADREX Corporation) with i.d. and film thickness of 0.25 mm and 0.05 µm, respectively, and connected to an Agilent 5973 quadrupole mass selective detector. Samples were injected in splitless mode (5 min) with the injector operating at 220ºC. The GC oven temperature program was set at an initial temperature of 75ºC, raised to 163ºC at 15ºC/min, then raised to 173ºC at 3ºC/min, holding for 3 min, then raised to 175ºC at 1ºC/min, and then raised to 250ºC at 15ºC/min, holding for 5 min. The flow rate of the carrier gas (He) was set at 1.7 mL/min. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 40–500 *m/z*, in a full scan acquisition mode. Identification of the chromatogram peaks was done comparing all mass spectra with the database system of the GC–qMS equipment (Wiley 275). FAs quantification was achieved with the internal standard method using $C_{17:0}$ and considering the response factor of all the identified FAs as one. One aliquot of each sea salt extract was analysed.

2.12. DATA ANALYSIS

Volatile components

Hierarchical cluster analysis (HCA), using Ward's method (agglomerative hierarchical clustering procedure), was applied with the aim of helping to characterise the data set. HCA is an exploratory tool designed to reveal natural groupings (or clusters) within a data set, by means of a dendrogram (tree diagram), which would otherwise not be apparent. A full data set comprising 165 volatile components (variables) and a sub-set of ten compounds common to all sea salts under study were considered. Each data set consisted of 21 observations, i.e. 3 replicate analyses from 7 sea salts. HCA was applied to auto-scaled GC peak areas. Autoscaling is a data pre-treatment process that makes variables of different scales comparable. Each variable is autoscaled separately by subtracting its mean value and dividing by its standard deviation.

A heatmap visualization of the full data set (7 sea salts and 165 volatile components), normalized by applying a logarithm function, was also performed.

Polymeric material

A first approach on the characterisation of the PM isolated from sea salt was a mid-infrared spectroscopy analysis, as described in section 2.6. The resulting spectra were transferred through JCAMP-DX format into the data analysis software developed in the Institut National Agronomique Paris-Grignon in collaboration with the University of Aveiro (Barros, 1999). Principal Component Analysis (PCA) was applied to the auto-scaled mid-infrared spectra (1800-700 cm^{-1} at 8 cm^{-1} resolution with 128 co-added scans) of the PM from the 16 Atlantic Ocean salts under study, each with five independent replicates, in order to extract the main sources of variability.

HCA, heatmap and PCA were performed using the R (version 2.12.0) statistical software package (R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing; Vienna, Austria, 2009).

III.A. HEADSPACE SOLID-PHASE MICROEXTRACTION COMBINED WITH COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY TIME-OF-FLIGHT MASS SPECTROMETRY FOR THE DETERMINATION OF VOLATILE COMPOUNDS FROM MARINE SALT

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Headspace solid-phase microextraction combined with comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry for the determination of volatile compounds from marine salt

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In this work, a methodology to characterise the volatile and semi-volatile compounds from sea salt by headspace solid-phase microextraction (HS-SPME) and comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC–ToFMS) was developed. Samples from two saltpans of Aveiro, in Portugal, with diverse locations, obtained over three years (2004, 2005, and 2007) were analysed. A 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane SPME fibre was used. The volatiles present in the headspace of the solid salt samples (crystals) were equilibrated overnight at 60ºC and extracted for 60 min prior to injection in the GC×GC–ToFMS. 157 compounds, distributed over the chemical groups of hydrocarbons, aldehydes, esters, furans, haloalkanes, ketones, ethers, alcohols, terpenoids, C_{13} norisoprenoids, and lactones were detected across the samples. Furans, haloalkanes and ethers were identified for the first time in sea salt. The large number of co-elutions on the first column that were resolved by the GC×GC system revealed the complexity of sea salt volatile composition. The existence of a structured 2D chromatographic behaviour according to volatility, in the first dimension (^{1}D), and primarily polarity, in the second dimension (^{2}D), was demonstrated, allowing more reliable identifications. The resolution and sensitivity of GC×GC–ToFMS enabled the separation and identification of a higher number of volatile compounds compared to GC–qMS, allowing a deeper characterisation of this natural product.

III.A.1. CHROMATOGRAM CONTOUR PLOT ANALYSIS

The volatile composition of sea salt from two saltpans of Aveiro with distinct locations, *Peijota* (PJ) and *18 dos Caramonetes* (18C), obtained over three years (2004, 2005, and 2007) was analysed by GC×GC–ToFMS. The GC×GC analysis was performed on a system comprising a non-polar (nP) thick-film ^{1}D column and a ^{2}D column containing a thin-film BP20 polar (P) stationary phase. This column combination provided two almost independent separations (orthogonality). On the nP column, analytes were separated according to their vapour pressure/volatility, and on the P column, analytes were separated according to their polarity.

Automated processing of HS-SPME/GC×GC–ToFMS data was used to tentatively identify all peaks in the GC×GC chromatogram contour plots with signal-to-noise threshold >100. The peak table generated automatically by ChromaTOF software was further examined, and identification was confirmed or changed based on criteria described in section 2.3 (Chapter II). **Table 3.1** summarizes the information obtained for each compound tentatively identified in the analysis of sea salt, including GC peak areas, RSD, and RIs experimentally calculated and available in the literature for a 5% phenyl polysilphenylene-siloxane phase (or its equivalent) column. A maximum difference of 30–40 ($|RI_{calc}-RI_{lit}|$) was achieved. This difference in RI is considered reasonable (<5%) if one takes into account that: (i) the literature values were determined in a one-dimensional chromatographic separation system, and the modulation causes some inaccuracy in first dimension retention time and (ii) the literature data is obtained from a large range of GC stationary phases (several commercial GC columns are composed of 5% phenyl polysilphenylene-siloxane or equivalent stationary phases), which had a slightly different separation selectivity than BPX5.

Fig. 3.1 shows an example of a GC×GC–ToFMS chromatogram contour plot of the volatile composition of sea salt from Peijota saltpan from 2007 (PJ07), presenting the larger number of identified compounds (101). More intense peaks are numbered according to **Table 3.1**, and some examples of bands and an additional cluster formed by structurally related terpenoid compounds are also indicated (**Fig. 3.1**). The *n*-alkanes series (C_9–C_{20}) used for the calculation of experimental RIs is also superimposed on the contour plot.

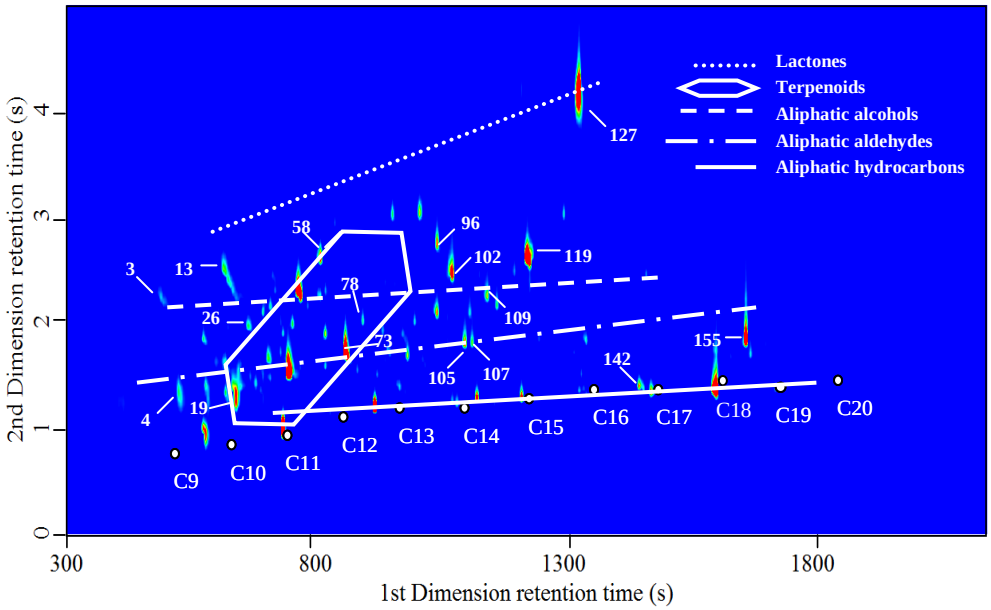

Figure 3.1. GC×GC contour plot of the volatile composition of sea salt from saltpan Peijota 2007. The white spots indicate the position of the series of alkanes (C_9-C_{20}). Bands and cluster formed by structurally related compounds are indicated (attribution of peak numbers shown in **Table 3.1**).

The structured GC×GC (2D) chromatographic behaviour demonstrated in **Fig. 3.1** is explained by the differences in volatility, and especially in polarity among the chemical groups identified. For a better understanding of **Fig. 3.1**, part of the identified chemical groups was included to elucidate the principle of structured 2D chromatogram. Aliphatic hydrocarbons are the least polar compounds, presenting the lower retention time for the second dimension (^2t_R), and lactones, with higher polarity, presenting higher ^2t_R value. This can also be confirmed by the data reported in **Table 3.1** (^1t_R and ^2t_R). **Fig. 3.1** also shows that aliphatic aldehydes appear at higher ^2t_R than the hydrocarbons, followed by aliphatic alcohols. The chemical group of terpenoids (C_{10} monoterpenoids), that includes hydrocarbons, alcohols, aldehydes, and ketones, are represented by a polygon within the 2D space. The sesquiterpenoid spathulenol (peak 132) is not included in this polygon,

Table 3.1. Volatile compounds identified by GC×GC–ToFMS in sea salt from two saltpans of Aveiro (*Peijota* – **PJ** and *18 dos Caramonetes* – **18C**) obtained over three years (2004, 2005, and 2007).

Peak N°	^1t_R[a]	^2t_R[a]	Compound	RI_{lit}[b]	RI_{calc}[c]	PJ04		PJ05		PJ07		18C04		18C05		18C07	
						Peak area[d] ($\times10^{-5}$) and R.S.D.(%)											
			Hydrocarbons														
			Aliphatics														
28	680	1.73	3-Ethyl-2-methyl-1,3-hexadiene	-	1052	-	-	-	-	0.42	(24)	-	-	-	-	-	-
32	695	1.55	C_{10} isomer	-	1065	3.53	(6)	1.94	(26)	3.64	(9)	-	-	-	-	-	-
47	735	1.04	Undecane	1100	1101	-	-	1.38	(64)	-	-	-	-	-	-	-	-
64	840	1.01	C_{11} isomer	-	1196	-	-	-	-	1.01	(33)	-	-	-	-	-	-
70	845	1.14	Dodecane	1200	1201	-	-	8.58	(29)	-	-	-	-	4.69[f]	(4)	-	-
87	955	1.22	C_{12} isomer	-	1293	-	-	-	-	1.48	(18)	-	-	1.57	(46)	-	-
88	960	1.19	Tridecane	1300	1297	1.04[f]	(5)	13.95	(40)	-	-	1.46	(15)	5.38	(58)	-	-
92	980	1.18	C_{13} isomer	-	1312	0.66[f]	(3)	1.69	(25)	-	-	-	-	-	-	-	-
94	1010	1.19	C_{13} isomer	-	1336	-	-	1.89	(65)	-	-	-	-	-	-	-	-
97	1050	1.16	2,6,10-Trimethyl-dodecane	-	1366	0.76	(42)	-	-	-	-	-	-	-	-	-	-
98	1055	1.24	C_{13} isomer	-	1370	-	-	2.40	(45)	-	-	-	-	-	-	-	-
103	1090	1.28	Tetradecane	1400	1397	-	-	3.17	(40)	-	-	-	-	-	-	-	-
110	1160	1.27	C_{14} isomer	-	1449	1.72	(32)	1.46	(47)	1.31	(20)	-	-	-	-	-	-
112	1190	1.26	C_{14} isomer	-	1471	-	-	-	-	-	-	-	-	-	-	3.58	(47)
114	1215	1.31	C_{14} isomer	-	1490	-	-	-	-	-	-	-	-	-	-	3.14	(25)
121	1250	1.28	C_{15} isomer	-	1516	-	-	-	-	-	-	-	-	-	-	2.68	(44)
123	1280	1.27	C_{15} isomer	-	1539	1.19	(67)	-	-	-	-	-	-	-	-	-	-
125	1295	1.33	C_{15} isomer	-	1551	1.07	(67)	-	-	-	-	-	-	-	-	-	-
131	1345	1.35	C_{15} isomer	-	1590	-	-	-	-			-	-	-	-	1.72	(9)
136	1405	1.32	C_{16} isomer	-	1637	2.26	(40)	-	-	1.67	(23)	-	-	-	-	-	-
137	1410	1.37	2,6,10-Trimethyl-pentadecane	1629	1641	-	-	1.04	(33)	-	-	-	-	-	-	-	-
142	1460	1.45	1-Heptadecene	-	1681	-	-	18.88	(30)	16.22	(7)	-	-	-	-	-	-
143	1465	1.32	C_{16} isomer	-	1685	-	-	-	-	-	-	-	-	-	-	4.36[f]	(24)
145	1475	1.10	Heptadecane	1700	1693	-	-	-	-	-	-	0.90	(17)	-	-	5.30	(17)
146	1530	1.36	C_{17} isomer	-	1731	2.80	(67)	-	-	-	-	-	-	-	-	2.20	(32)
149	1555	1.35	C_{17} isomer	-	1748	0.72[f]	(66)	-	-	-	-	-	-	-	-	-	-
150	1565	1.38	C_{17} isomer	-	1754	0.81[f]	(18)	-	-	-	-	-	-	-	-	-	-
151	1605	1.32	2,6,10,14-tetramethyl-hexadecane	-	1781	9.56	(5)	2.76	(34)	16.29[f]	(8)	-	-	2.47[f]	(14)	6.89	(4)
156	1725	1.36	C_{18} isomer	-	1879	-	-	1.06	(15)	1.28	(16)	-	-	-	-	-	-
			Aromatics														
2	490	1.35	Xylene	888	873	0.52[f]	(58)	0.31[e]	-	-	-	-	-	-	-	-	-

100	1065	1.87	1,4-Dimethyl-2,5-bis(1-methylethyl)benzene	-	1374	0.48	(28)	0.70	(42)	-	-	-	-	-	-	-	-
122	1275	1.68	1-Butylhexylbenzene	-	-	-	-	-	-	-	-	-	-	-	-	0.17[e]	-
126	1315	1.69	1-Ethyloctylbenzene	-	-	-	-	-	-	-	-	-	-	-	-	0.10[e]	-
135	1400	1.70	1-Butylheptylbenzene	-	1633	0.29[f]	(93)	-	-	-	-	-	-	-	-	-	-
139	1415	1.72	1-Propyloctylbenzene	1636	1645	-	-	-	-	-	-	-	-	-	-	0.40	(42)
141	1430	1.73	1,1-Dimethyldecylbenzene	-	1657	0.61[e]	-	-	-	-	-	-	-	-	-	-	-
147	1530	1.70	1-Butyloctylbenzene	-	1731	0.48[f]	(105)	-	-	-	-	-	-	-	-	1.13	(28)
148	1545	1.71	1-Propylnonylbenzene	-	1741	0.22[e]	-	-	-	-	-	-	-	-	-	0.26	(29)
			Subtotal (GC Peak area)			**20.11**	**(39)**	**61.00**	**(31)**	**37.89**	**(23)**	**2.36**	**(11)**	**11.72**	**(67)**	**31.76**	**(21)**
			Subtotal (%)			**6.90**	**(41)**	**29.28**	**(23)**	**4.59**	**(26)**	**8.88**	**(10)**	**11.91**	**(57)**	**12.51**	**(14)**
			Aldehydes														
			Aliphatics														
1	430	1.26	Hexanal	805	815	1.55[e]	-	-	-	-	-	-	-	-	-	-	-
4	525	1.35	Heptanal	916	906	10.74	(44)	-	-	27.89	(18)	-	-	-	-	-	-
8	595	1.67	2-Heptenal	951	973	-	-	-	-	1.88[e]	-	-	-	-	-	-	-
19	640	1.37	Octanal	1012	1015	13.41[f]	(2)	6.24[f]	(49)	45.80	(30)	-	-	4.97	(17)	-	-
26	670	1.94	2,4-Heptadienal	1009	1043	0.49[e]	-	-	-	1.33	(45)	-	-	-	-	-	-
51	745	1.50	Nonanal	1127	1111	32.86[f]	(19)	14.84[f]	(42)	82.88	(13)	1.27[f]	(43)	12.63	(29)	2.44	(25)
60	815	1.88	2-Nonenal	-	1174	0.78[e]	-	-	-	1.49	(5)	-	-	-	-	-	-
73	860	1.70	Decanal	1216	1214	46.60[f]	(45)	11.66[f]	(62)	88.32	(24)	2.23[f]	(18)	25.16	(23)	-	-
79	895	2.01	2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde	1223	1243	2.76	(3)	1.28	(52)	4.25	(9)	-	-	-	-	-	-
83	935	1.95	2-Decenal	1261	1277	0.79	(56)	0.45[e]	-	2.90	(31)	-	-	-	-	-	-
93	985	1.70	Undecanal	1316	1317	3.13[f]	(45)	0.43[f]	(25)	7.43	(10)			1.25	(17)	-	-
107	1115	1.84	Dodecanal	1411	1416	2.66[f]	(33)	1.13[f]	(7)	6.08	(10)	0.63[e]	-	1.28	(8)	0.44	(5)
120	1245	1.90	Tridecanal	1523	1513	0.60[f]	(12)	-	-	2.20	(8)	-	-	-	-	-	-
133	1380	1.91	Tetradecanal	1611	1618	1.15[e]	-	1.12	(64)	1.64	(14)	-	-	-	-	-	-
153	1665	2.19	5,9,13-Trimethyl-4,8,12-tetradecatrienal	-	1828	-	-	-	-	0.97	(16)	-	-	-	-	0.32	(32)
			Aromatics														
13	615	2.54	Benzaldehyde	986	993	-	-	3.02	(50)	36.63	(11)	7.70	(20)	-	-	-	-
35	700	2.76	Benzeneacetaldehyde	-	1071	0.19[e]	-	-	-	0.79	(16)	-	-	-	-	-	-
			Subtotal (GC Peak area)			**81.85**	**(66)**	**28.44**	**(79)**	**311.22**	**(11)**	**10.25**	**(37)**	**45.29**	**(23)**	**3.19**	**(20)**
			Subtotal (%)			**26.78**	**(59)**	**13.68**	**(71)**	**37.42**	**(8)**	**37.59**	**(27)**	**49.94**	**(4)**	**1.25**	**(9)**
			Esters														
			Aliphatics														
23	670	1.31	Methyl 2-ethylhexanoate	1029	1042	-	-	-	-	-	-	-	-	-	-	4.33	(6)
63	835	1.99	Butyl 2,4-dimethyl-2-nitro-4-pentenoate	-	1193	-	-	-	-	-	-	-	-	0.51[f]	(28)	-	-

75	880	2.10	Dimethyl tetramethylsuccinate	-	1231	-	-	-	-	-	-	-	-	0.28[f]	(27)	-	-
96	1045	2.74	1-Hydroxy-2,4,4-trimethylpentan-3-yl 2-methylpropanoate	-	1364	1.83	(51)	1.44[f]	(49)	11.30	(21)	-	-	-	-	8.40	(3)
102	1075	2.50	3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	1381	1387	3.94	(41)	8.04	(64)	24.12	(22)	1.13	(49)	1.91	(17)	16.36	(7)
129	1340	1.89	2,4,4-Trimethylpentane-1,3-diyl bis(2-methylpropanoate)	-	1586	-	-	-	-	6.22	(17)	-	-	-	-	3.21	(17)
134	1385	1.69	Isopropyl laurate	1618	1621	-	-	-	-	1.98	(18)	-	-	-	-	-	-
152	1635	1.64	Isopropyl myristate	-	1801	-	-	0.48[e]	-	0.37[f]	(11)	-	-	-	-	-	-
157	1870	1.72	Isopropyl palmitate	2011	2011	-	-	-	-	1.28	(22)	-	-	-	-	-	-
			Aromatics														
124	1290	3.03	Ethyl 4-ethoxybenzoate	1522	1549	-	-	-	-	-	-	0.32[f]	(17)	0.37	(25)	-	-
155	1705	3.12	Dibutyl phthalate	1897	1864	-	-	22.65[f]	(139)	0.41	(24)	0.22[f]	(9)	0.69	(20)	-	-
			Subtotal (GC Peak area)			**5.77**	**(44)**	**24.26**	**(129)**	**45.55**	**(19)**	**1.49**	**(26)**	**3.49**	**(22)**	**32.30**	**(2)**
			Subtotal (%)			**2.04**	**(50)**	**13.37**	**(138)**	**5.48**	**(18)**	**5.75**	**(39)**	**3.88**	**(11)**	**12.87**	**(10)**
			Furans														
14	620	1.34	2-Pentylfuran	999	997	0.83	(21)	1.08	(67)	8.50	(64)	-	-	-	-	-	-
44	730	1.40	2-Hexylfuran	-	1097	-	-	-	-	1.20	(14)	-	-	-	-	-	-
65	840	1.47	2-Heptylfuran	-	1197	-	-	0.14[e]	-	0.50	(15)	-	-	-	-	-	-
89	960	1.64	2-Octylfuran	-	1297	-	-	0.15[e]	-	0.34	(10)	-	-	-	-	-	-
			Subtotal (GC Peak area)			**0.83**	**(21)**	**1.17**	**(71)**	**10.53**	**(54)**	-	-	-	-	-	-
			Subtotal (%)			**0.29**	**(26)**	**0.54**	**(68)**	**1.25**	**(51)**	-	-	-	-	-	-
			Haloalkanes														
31	695	1.25	1-Chloro-octane	1044	1065	0.33[f]	(24)	1.47[f]	(78)	2.32	(57)	-	-	-	-	1.14	(38)
55	790	1.41	1-Bromo-octane	-	1151	-	-	-	-	0.48	(14)	-	-	-	-	-	-
57	810	1.41	1-Chloro-nonane	1159	1170	1.08[f]	(55)	-	-	0.78	(21)	-	-	0.30[f]	(36)	0.33	(21)
			Subtotal (GC Peak area)			**0.94**	**(59)**	**1.47**	**(78)**	**3.58**	**(43)**	**-**	**-**	**0.30**	**(36)**	**1.47**	**(34)**
			Subtotal (%)			**0.33**	**(67)**	**0.67**	**(80)**	**0.43**	**(39)**	**-**	**-**	**0.29**	**(37)**	**0.58**	**(25)**
			Ketones														
			Aliphatics														
6	580	1.43	6-Methyl-2-heptanone	-	958	72.51	(7)	23.49	(37)	12.05	(25)	3.95	(18)	-	-	-	-
7	580	2.24	2-Cyclohexen-1-one	914	959	-	-	-	-	0.67	(2)	-	-	-	-	-	-
10	605	1.62	2-Methyl-1-hepten-6-one	966	983	2.87	(12)	0.42	(60)	1.96	(47)	-	-	-	-	0.24	(49)
15	620	1.61	6-Methyl-5-hepten-2-one	1003	997	10.10	(33)	18.75[f]	(34)	10.29	(19)	1.09[f]	(22)	3.23	(10)	6.46[f]	(6)
20	645	1.61	2,4,4-Trimethyl-cyclopentanone	-	1020	6.19	(18)	3.12	(39)	7.29	(8)	-	-	-	-	-	-
25	670	1.49	1-(2,2-Dimethylcyclopentyl)-ethanone	-	1042	1.24	(36)	1.46	(41)	2.43	(37)	-	-	-	-	-	-
27	680	1.45	2,2,6-Trimethyl-cyclohexanone	1036	1051	6.68	(4)	3.79	(27)	5.10	(10)	-	-	0.47[e]	-	2.15	(4)
29	685	1.68	3,5,5-Trimethyl-3-cyclohexen-1-one	-	1056	-	-	0.34[e]	-	0.97[f]	(2)	-	-	-	-	0.61	(30)

30	690	2.17	2,3-Dimethyl-2-cyclopenten-1-one	-	1061	-	-	-	-	0.34	(9)	-	-	-	-	0.47	(20)
37	705	1.67	3,6,6-Trimethyl-cyclohexen-2-enone	-	1074	10.24	(7)	2.82	(39)	8.10	(17)	0.31	(14)	-	-	1.45	(5)
38	705	2.14	1-(2-Methyl-1-cyclopenten-1-yl)-ethanone	-	1075	-	-	-	-	0.47	(24)	-	-	-	-	0.90	(20)
42	715	2.49	3-Methyl-2-cyclohexen-1-one	-	1084	0.71	(29)	-	-	3.56	(10)	-	-	0.29	(13)	3.17	(19)
45	730	1.55	2-Nonanone	1083	1097	1.35	(48)	1.34	(47)	1.23	(7)	-	-	0.47	(46)	2.17	(19)
46	730	1.74	3,4,4-Trimethyl-2-cyclohexen-1-one	1097	1097	2.81	(7)	0.64[f]	(29)	3.32	(19)	-	-	-	-	-	-
52	745	1.75	2,7,7-Trimethylbicyclo[3.1.1]hept-2-en-6-one	-	1111	-	-	1.72[e]	-	-	-	-	-	-	-	-	-
53	750	1.67	C_9 (*m/z* 41, 69, 55, 98, 42)	-	1115	-	-	3.77	(18)	-	-	-	-	-	-	-	-
54	785	2.18	3,5,5-Trimethyl-2-cyclohexen-1-one	1138	1147	0.56	(9)	-	-	2.72	(14)	-	-	0.40[e]	-	2.12	(11)
56	805	2.11	6,6-Dimethyl-bicyclo[3.1.1]heptan-2-one	1142	1166	-	-	0.22[f]	(19)	0.47	(43)	-	-	-	-	-	-
58	810	2.64	Ketoisophorone	1169	1171	6.65	(18)	3.71	(36)	18.90	(10)	2.30	(19)	3.72	(9)	30.55	(16)
62	830	1.49	3,3,4,4-Tetramethyl-2-pentanone	-	1188	0.15[f]	(24)	-	-	-	-	-	-	-	-	-	-
66	840	1.64	2-Decanone	1190	1197	1.14	(67)	-	-	1.48	(9)	-	-	-	-	0.57	(26)
69	840	2.98	2,2,6-Trimethyl-1,4-cyclohexanedione	1196	1198	1.40	(50)	1.17[f]	(11)	2.80	(7)	0.57	(28)	3.49	(11)	5.33	(21)
71	855	1.40	2,6,8-Trimethyl-4-nonanone	-	1210	0.34[e]	-	-	-	-	-	-	-	-	-	-	-
77	890	2.31	1,3,3-Trimethyl-2-Oxabicyclo[2.2.2]octan-6-one	-	1239	-	-	-	-	-	-	-	-	-	-	0.45	(17)
82	925	1.50	C_{11} (*m/z* 122, 43, 109, 41, 135)	-	1268	1.18	(21)	1.88	(43)	2.03	(11)	-	-	-	-	-	-
91	965	1.78	2-Undecanone	1294	1301	0.54	(14)	-	-	0.69	(10)	-	-	-	-	-	-
95	1010	3.07	2-(2-Methylpropylidene)-cycloheptanone	-	1337	1.53	(24)	-	-	-	-	-	-	-	-	-	-
99	1060	1.87	4-(2,4,4-Trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one	-	1374	0.48	(28)	0.70	(42)	0.39	(16)	-	-	-	-	-	-
104	1090	1.82	2-Dodecanone	-	1398	0.25[e]	-	-	-	0.42	(14)	-	-	-	-	-	-
105	1100	1.79	6,10-Dimethyl-2-undecanone	1410	1405	14.43	(9)	3.77	(57)	11.79	(15)	-	-	-	-	-	-
108	1130	2.38	6-Methyl-6-(5-methylfuran-2-yl)heptan-2-one	-	1428	0.57	(16)	1.03	(46)	1.13	(22)	-	-	-	-	0.51	(6)
115	1215	2.08	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	1490	1490	-	-	0.51[f]	(47)	-	-	-	-	-	-	-	-
118	1225	1.90	2-Tridecanone	-	1498	-	-	0.21[e]	-	0.34	(10)	-	-	-	-	-	-
154	1670	1.93	6,10,14-Trimethyl-2-pentadecanone	1843	1832	3.99	(53)	2.97[f]	(55)	43.01	(21)	1.16	(25)	5.95	(23)	4.55	(21)
			Aromatic														
43	725	2.70	Acetophenone	1065	1093	0.64	(53)	-	-	1.06	(17)	-	-	-	-	0.53	(45)
			Subtotal (GC Peak area)			**148.14**	**(10)**	**68.32**	**(44)**	**144.70**	**(11)**	**9.01**	**(5)**	**17.70**	**(7)**	**60.06**	**(6)**
			Subtotal (%)			**51.25**	**(20)**	**32.24**	**(36)**	**17.42**	**(9)**	**34.03**	**(9)**	**20.16**	**(22)**	**23.90**	**(9)**

			Ethers														
5	560	1.53	1-Methoxy-4-methyl-benzene	-	940	-	-	-	-	1.19	(66)	-	-	-	-	-	-
80	895	3.58	2,4,8,10-Tetraoxaspiro[5.5]undecane	-	1245	-	-	-	-	-	-	-	-	-	-	7.70[f]	(108)
140	1430	1.48	Dioctyl ether	-	1657	-	-	-	-	0.96	(8)	-	-	-	-	0.42	(28)
			Subtotal (GC Peak area)			-	-	-	-	-	-	-	-	-	-	**8.12**	**(91)**
			Subtotal (%)			-	-	-	-	-	-	-	-	-	-	**3.21**	**(106)**
			Sulfur compound														
16	620	1.81	Dimethyl trisulfide	-	997	0.64[f]	(38)	-	-	-	-	-	-	-	-	-	-
			Subtotal (GC Peak area)			**0.23**	**(24)**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
			Subtotal (%)			**0.08**	**(34)**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
			Alcohols														
			Aliphatics														
3	490	2.24	1-Hexanol	868	874	-	-	-	-	11.27	(19)	-	-	-	-	-	-
11	605	2.11	1-Heptanol	-	983	0.36[e]	-	-	-	2.56	(20)	-	-	-	-	-	-
12	615	2.04	1-Octen-3-ol	978	992	-	-	-	-	0.92	(52)	-	-	-	-	-	-
21	665	1.96	2-Ethyl-1-hexanol	1029	1038	5.61	(67)	1.93	(66)	4.24	(46)	0.45	(38)	3.62	(21)	34.18	(61)
33	695	2.14	2,4,4-Trimethylcyclohexa-2-en-1-ol	-	1066	2.16	(5)	0.46[f]	(60)	5.16	(10)	-	-	-	-	-	-
34	700	2.19	C_9 (*m/z* 43, 84, 69, 41, 81)	-	1070	-	-	-	-	1.51[f]	(1)	-	-	-	-	-	-
40	710	2.18	1-Octanol	1080	1079	1.97	(46)	-	-	7.83	(18)	-	-	0.72[f]	(60)	-	-
48	735	2.35	1-Undecyn-4-ol	-	1102	0.54	(40)	-	-	-	-	-	-	-	-	-	-
61	820	2.33	1-Nonanol	1180	1179	2.61	(64)	-	-	5.36	(3)	-	-	0.78	(39)	-	-
84	940	2.33	1-Decanol	1266	1281	0.99	(55)	-	-	1.68	(22)	-	-	-	-	-	-
90	960	2.38	4,8-Dimethyl-1-nonanol	1276	1298	0.50	(7)	-	-	1.29	(34)	-	-	-	-	-	-
101	1075	1.31	1-Undecanol	1370	1386	-	-	0.46	(29)	0.40[f]	(20)	-	-	0.26[e]	-	-	-
106	1110	2.09	2-Dodecanol	1387	1413	0.61	(49)	-	-	3.26	(8)	-	-	-	-	-	-
113	1200	2.50	1-Dodecanol	-	1480	-	-	-	-	3.84	(13)	-	-	-	-	-	-
128	1340	1.41	1-Tridecanol	-	1586	-	-	-	-	0.61[f]	(10)	-	-	-	-	-	-
138	1410	2.36	3,7,11-Trimethyl-1-dodecanol	-	1642	-	-	-	-	0.61[f]	(6)	-	-	-	-	-	-
144	1465	2.46	1-Tetradecanol	1673	1686	1.48[e]	-	-	-	4.62	(7)	-	-	-	-	1.53	(17)
			Aromatic														
50	740	3.35	Dimethylbenzenemethanol	1080	1108	0.65	(30)	0.69	(78)	0.70	(33)	-	-	0.16	(5)	0.87	(17)
			Subtotal (GC Peak area)			**16.24**	**(48)**	**3.38**	**(60)**	**54.80**	**(9)**	**0.45**	**(38)**	**5.13**	**(31)**	**36.58**	**(57)**
			Subtotal (%)			**5.73**	**(58)**	**1.57**	**(54)**	**6.59**	**(3)**	**1.68**	**(31)**	**5.69**	**(22)**	**14.03**	**(51)**
			Terpenoids														
22	670	1.26	1,8-Cineole	1032	1042	-	-	-	-	4.58[f]	(37)	-	-	-	-	-	-
24	670	1.33	Cymene	1038	1042	-	-	0.22[f]	(4)	-	-	-	-	-	-	-	-
39	710	1.80	Dihydromyrcenol	1072	1079	0.31	(2)	-	-	0.26	(14)	-	-	-	-	-	-
49	740	2.01	Linalool	1098	1106	0.30[f]	(16)	-	-	0.37	(11)	-	-	-	-	0.29[e]	-

59	815	1.85	Camphor	1143	1174	0.53	(18)	-	-	-	-	-	-	-	-	0.38	(8)
67	840	2.14	Isomenthol	1178	1197	0.81	(17)	-	-	0.51	(41)	-	-	-	-	0.61	(7)
68	840	2.47	Borneol	1166	1198	-	-	-	-	0.25	(8)	-	-	-	-	-	-
72	855	3.45	Cymen-8-ol	1183	1211	-	-	-	-	-	-	-	-	-	-	0.06[e]	-
74	870	2.19	Safranal	1197	1223	-	-	-	-	0.55	(40)	-	-	-	-	-	-
76	885	2.45	Verbenone	1204	1235	0.62[e]	-	-	-	-	-	-	-	-	-	-	-
78	895	1.96	β-Cyclocitral	1223	1243	-	-	-	-	4.25	(9)	-	-	-	-	1.07	(1)
81	905	2.50	Eucarvone	1248	1252	-	-	-	-	0.53	(7)	-	-	-	-	-	-
85	940	2.40	Carvone	1242	1281	-	-	0.39[f]	(35)	-	-	-	-	-	-	-	-
132	1365	2.41	Spathulenol	1619	1606	-	-	-	-	-	-	1.24	(31)	-	-	-	-
			Subtotal (GC Peak area)			**2.06**	**(20)**	**0.61**	**(21)**	**9.78**	**(33)**	**1.24**	**(31)**	-	-	**2.18**	**(6)**
			Subtotal (%)			**0.72**	**(30)**	**0.27**	**(24)**	**1.16**	**(29)**	**4.79**	**(43)**	-	-	**0.87**	**(6)**
			C_{13} Norisoprenoids														
109	1145	2.28	α-Ionone	1426	1439	1.85	(20)	1.34	(36)	7.76	(7)	-	-	-	-	5.55	(23)
111	1160	2.13	Dihydro-β-ionone	-	1450	-	-	-	-	-	-	-	-	-	-	1.50	(15)
117	1220	2.38	β-Ionone	1485	1494	-	-	0.28	(42)	0.48	(9)	-	-	-	-	1.24	(11)
119	1225	2.63	β-Ionone-5,6-epoxide	1463	1498	5.30	(54)	3.11	(46)	59.05	(4)	0.81	(30)	-	-	25.04	(14)
			Subtotal (GC Peak area)			**7.15**	**(45)**	**4.72**	**(43)**	**67.28**	**(4)**	**0.81**	**(30)**	-	-	**33.33**	**(12)**
			Subtotal (%)			**2.53**	**(55)**	**2.23**	**(34)**	**8.11**	**(5)**	**3.14**	**(42)**	-	-	**13.18**	**(3)**
			Lactones														
9	595	3.39	5,5-Dimethyl-2(5H)-furanone	951	975	-	-	-	-	0.35	(3)	-	-	-	-	0.33[f]	(14)
17	630	2.82	Dihydro-5,5-dimethyl-2(3H)-furanone	992	1007	0.65	(22)	2.82	(40)	1.72	(8)	-	-	-	-	0.60	(18)
18	635	2.65	Dihydro-3,3-dimethyl-2(3H)-furanone	-	1012	-	-	0.43	(44)	-	-	-	-	0.33	(3)	-	-
36	700	2.97	5-Ethenyldihydro-5-methyl-2(3H)-furanone	-	1071	-	-	1.04[f]	(50)	-	-	-	-	-	-	-	-
41	710	2.87	6-Allyltetrahydropyran-2-one	-	1080	-	-	-	-	0.54	(5)	-	-	-	-	-	-
86	945	3.46	5-Butyldihydro-2(3H)-furanone	-	1286	-	-	-	-	0.34	(39)	-	-	-	-	-	-
116	1215	4.23	7a-Methyl-3-methylenehexahydro benzofuran-2-one	-	1492	-	-	-	-	2.30	(14)	-	-	-	-	-	-
127	1330	4.26	Dihydroactinidiolide (isomer)	-	1580	9.55[f]	(16)	4.88	(43)	138.04	(6)	1.07	(32)	4.88	(31)	45.73	(25)
130	1340	4.05	Dihydroactinidiolide (isomer)	-	1588	-	-	7.23[f]	(22)	-	-	-	-	2.99[f]	(24)	-	-
			Subtotal (GC Peak area)			**7.02**	**(78)**	**13.64**	**(38)**	**143.30**	**(6)**	**1.07**	**(32)**	**7.21**	**(5)**	**46.56**	**(25)**
			Subtotal (%)			**2.32**	**(79)**	**6.47**	**(29)**	**17.29**	**(9)**	**4.14**	**(42)**	**8.23**	**(23)**	**18.43**	**(22)**
			Total			**293.34**	**(12)**	**206.33**	**(14)**	**830.77**	**(6)**	**26.67**	**(12)**	**90.75**	**(23)**	**252.84**	**(11)**
			Number of identified compounds			**82**		**68**		**101**		**19**		**31**		**55**	

[a] Retention times in seconds (s) for first (^{1}tR) and second (^{2}tR) dimensions; [b] RI: retention index reported in the literature for 5% phenyl-dimethyl polysilphenylene-siloxane GC column or equivalents (Adams, 1995; NIST, 2005); [c] RI: retention index obtained through the modulated chromatogram; [d] Mean of three replicates; [e] The compound was detected in one replicate; [f] The compound was detected in two replicates

since with five extra carbons than monoterpenoids it has a higher $^{1}t_{R}$ (1365 s). By demonstrating that chemically-related compounds have related spatial distributions in 2D, a structured chromatogram contour plot allows more reliable identifications.

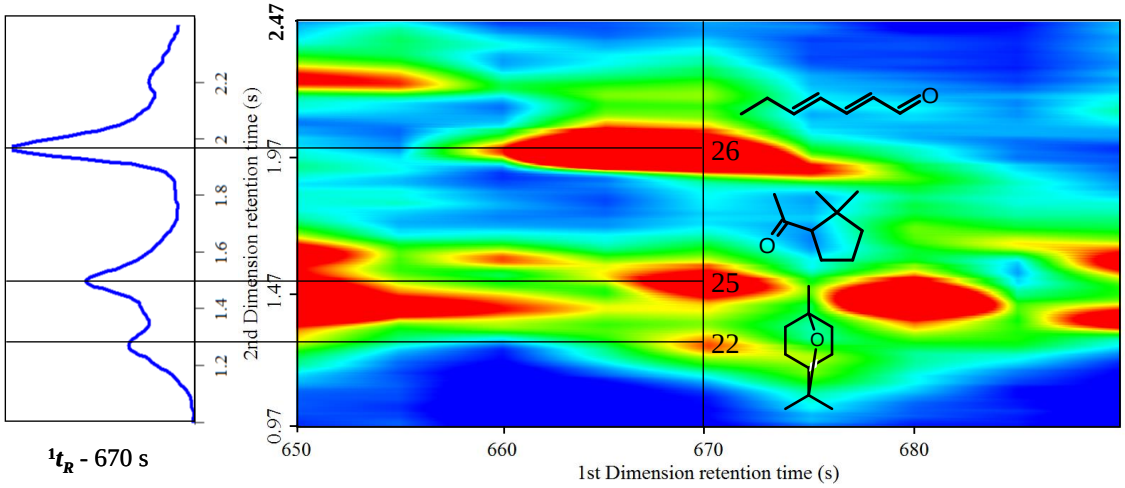

Figure 3.2. Blow-up of a part of the GC×GC contour plot presented in **Fig. 3.1**. Compounds numbered according to **Table 3.1**.

Fig. 3.2 shows an expansion of a part of the GC×GC chromatogram contour plot presented in **Fig. 3.1**, showing the usefulness of the ^{2}D column for resolution of selected volatile compounds from sea salt. The example of 1,8-cineole (peak 22) ($^{1}t_{R}$ =670 s, $^{2}t_{R}$ =1.26 s), 1-(2,2-dimethylcyclopentyl)-ethanone (peak 25) ($^{1}t_{R}$ =670 s, $^{2}t_{R}$ =1.49 s), and 2,4-heptadienal (peak 26) ($^{1}t_{R}$ =670 s, $^{2}t_{R}$ =1.94 s) was chosen. These compounds co-eluted on the BPX5 column, presenting the same $^{1}t_{R}$ (similar volatility), but exhibiting different polarities and, therefore, are separated on the BP20 ^{2}D column. Comparing the chemical structures of these three compounds, the different polarities observed are explained by the presence of carbonyl groups and double bonds. The carbonyl group of 1-(2,2-dimethylcyclopentyl)-ethanone and 2,4-heptadienal promote a higher polarity of these compounds compared to 1,8-cineole. The presence of two double bonds in addition to a carbonyl group can explain the proposed higher polarity of 2,4-heptadienal and consequently greater $^{2}t_{R}$. **Fig. 3.2** demonstrates an elucidative example where separation on the ^{2}D column arises for compounds with the same $^{1}t_{R}$. It is also important to point out that, in **Table 3.1**, the chemical groups of hydrocarbons, aldehydes, esters, and alcohols were

organized into aliphatic and aromatic compounds. The presence of an aromatic ring increases polarity of the molecules when compared with aliphatic chains; $^{2}t_{R}$ will always be greater for the aromatic compounds than for the respective aliphatic compounds on this nP/P column set (Tran *et al.*, 2006). Often data may be extracted from the structured 2D chromatogram contour plot for additional classification of unidentified compounds. In conclusion, the PJ07 chromatogram contour plot, reveals the full complexity of sea salt volatile composition, and demonstrates the importance of the GC×GC–ToFMS system for this type of analysis.

III.A.2. VOLATILE COMPOUNDS IDENTIFIED IN DIFFERENT SAMPLES OF SEA SALT

The analysis of the headspace volatile composition of sea salt by GC×GC–ToFMS allowed the identification of 157 volatile and semi-volatile organic compounds, almost four times the number of compounds identified in the previous study by GC–qMS (Silva *et al.*, 2009). These compounds are distributed over the chemical groups of hydrocarbons, aldehydes, esters, furans, haloalkanes, ketones, ethers, alcohols, terpenoids, C_{13} norisoprenoids, and lactones (**Table 3.1**); a sulfur compound (dimethyl trisulfide) apart from these chemical groups was also identified. Among all the chemical groups, ketones present the higher number of identified compounds (35), followed by hydrocarbons (30), alcohols (18), and aldehydes (17). One hundred and forty of these compounds were identified for the first time in sea salt. Among these, three chemical groups were identified for the first time: furans, haloalkanes, and ethers, although at lowest GC peak areas. With the exception of C_{13} norisoprenoids, the number of compounds in all chemical groups increased when compared to the results previously obtained (Silva *et al.*, 2009). The higher chromatographic separation, and increased sensitivity, limits of detection, and signal-to-noise ratio, are significantly enhanced by GC×GC–ToFMS compared to 1D-GC used previously (Silva *et al.*, 2009), which may explain the detection of a greater number of compounds. Furthermore, the different SPME coated fibre may also contribute to these results; the fibre used in this work, is noted to have higher extraction efficiency for furans, halogenated compounds, and ethers (Ferreira *et al.*, 2009; Klimánková *et al.*, 2008; Martendal *et al.*, 2007) when compared to CW/DVB previously used (Silva *et al.*, 2009).

Comparison of the results of sea salts obtained from each saltpan under study, *Peijota* (PJ) and *18 dos Caramonetes* (18C) allowed to verify that the number of compounds and total GC peak areas are always higher for PJ across all years. It was observed that of the salts produced in 2004, 2005, and 2007, for PJ and 18C, those harvested in 2007 exhibited the greatest number of compounds and total GC peak areas. For the PJ sea salt series, 82, 68, and 101 compounds were detected in samples produced in 2004, 2005, and 2007, respectively; for 18C, 19, 31, and 55 compounds were detected, respectively. For PJ, total GC peak areas decreased about three-fold from 2007 to 2005 and 2004: PJ07 (831×10^5); PJ04 (293×10^5); PJ05 (206×10^5). For 18C, total GC peak areas decreased from 18C07 (253×10^5) to 18C04 (27×10^5) (10-fold), and almost three-fold further for 18C05 (91×10^5). This tendency was also observed for all the chemical groups, with the exception of hydrocarbons and ketones in PJ, and aldehydes in 18C. Considering that all samples were analysed in the same period (October–December 2008), these results suggest that either loss of volatile compounds may occur with longer storage time, or it may be a real effect of simply less volatile content. As no significant differences were observed between salts from 2004 and 2005, storage may account for higher 2007 results. β-Ionone, a C_{13} norisoprenoid compound that exhibits a pleasant violet odour characteristic of Aveiro saltpans in samples of the year 2004 (Silva *et al.*, 2010), was only found in this study for samples of 2005 and 2007, supporting the hypothesis of loss of volatile compounds with storage, provided all samples originally had β-ionone.

Only nine from the total of 157 components of the volatile fraction of sea salt were detected in all the samples under study, indicating relatively high variability of this natural product, namely, nonanal, dodecanal, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate, 6-methyl-5-hepten-2-one, 6,10,14-trimethyl-2-pentadecanone, ketoisophorone, 2,2,6-trimethyl-1,4-cyclohexanedione, 2-ethyl-1-hexanol, and dihydroactinidiolide (isomer). The reproducibility ranged from 1% to 139%. In general, the volatile components of sea salt exhibit small areas, usually associated with a large variability. Furthermore, the high values of RSD obtained may also be explained by the fact that sea salt is a natural heterogeneous product, and its composition will depend strongly on environmental factors both for inputs and loss from the salt matrix.

The volatile components of sea salt seem to have several origins, such as algae, surrounding bacterial community, and anthropogenic activity (Silva *et al.*, 2009). Among

the 140 compounds identified for the first time in sea salt, some alcohols, aldehydes, esters, a ketone and a terpenol have previously been identified in algae: 1-hexanol, 1-octanol, hexanal, heptanal, benzaldehyde, octanal, 2,4-heptadienal, nonanal, tetradecanal, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate, isopropyl myristate, undecane, tridecane, heptadecane, 2-nonanone, and spathulenol (Beauchêne *et al.*, 2000; Elenkov *et al.*, 1995; Kamenarska *et al.*, 2006; Le Pape *et al.*, 2004; Shibata *et al.*, 2006; Tringali *et al.*, 1995). The compounds 1-heptanol, 1-nonanol, hexanal, heptanal, octanal, nonanal, dodecanal, tetradecanal, 3,5,5-trimethyl-2-cyclohexen-1-one, 2-nonanone, undecane, 1-heptadecene, and 2-pentylfuran were also identified in shore-dwelling cyanobacterial mat community (Evans, 1994). Compounds such as xylene, (1-butyloctyl)-benzene, and dibutyl phthalate, already identified as petroleum hydrocarbon contaminants in algae, may be reported as coming from anthropogenic activity (Erakin *et al.*, 2008). Thus, this study is in accordance with previously reported (Silva *et al.*, 2009) proposals for possible origins of the volatile components of sea salt.

III.A.3. CONCLUDING REMARKS

In this work a HS-SPME-GC×GC–ToFMS methodology was developed for the analysis of volatile and semi-volatile compounds from sea salt, applied to six samples obtained from two saltpans, and three harvests. 157 volatile compounds distributed over the chemical groups of hydrocarbons, aldehydes, esters, furans, haloalkanes, ketones, ethers, alcohols, terpenoids, C_{13} norisoprenoids, and lactones were detected. Furans, haloalkanes and ethers were identified for the first time in sea salt. Contour plot analysis revealed the complexity of sea salt volatile composition and confirmed the importance of a high resolution, sensitive analytical procedure (GC×GC–ToFMS) for this type of analysis. The structured 2D chromatographic profile arising from ^{1}D volatility and ^{2}D polarity was demonstrated, allowing more reliable identifications. ^2t_R values were consistently greater for aromatic than for aliphatic compounds, and aids classification of unidentified compounds.

Results obtained for analysis of salt from two diverse locations and harvests over three years suggest loss of volatile compounds according to storage duration of the salt,

with environmental factors surrounding the saltpans influencing the volatile composition of the salt. At present the relative contributions of these factors have not been quantified. Origins of newly identified compounds in sea salt are in accordance with previous propositions, with algae, surrounding bacterial community, and anthropogenic activity being obvious sources (Silva *et al.*, 2009).

The advantages of GC×GC–ToFMS over 1D-GC–qMS should contribute to easier and more reliable identification of compounds, and the search for origin-specific geographical chemical biomarkers, when comparing samples of sea salt from different locations.

III.B. CAN THE TYPICAL SURROUNDING MARINE ENVIRONMENT OF SALTPANS LEAD TO VOLATILE MARKERS OF SEA SALT?

Sea salt is a natural product that is obtained by evaporation of seawater in saltpans. These man-made systems can be located in different geographical areas, presenting different environmental surroundings. During the crystallization process, organic compounds from these surroundings can be incorporated into sea salt crystals, influencing their final composition. The aim of this chapter is to search for potential volatile markers of sea salt and to explore the relevance of geographical origin in the volatile composition of this natural product. Thus, sea salts from seven origins from France, Portugal, Continental Spain, Canary Islands, and Cape Verde, were analysed by headspace solid-phase microextraction combined with comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (HS-SPME/GC×GC–ToFMS). A total of 165 compounds were detected, ranging from 32 to 71 compounds per salt sample. The volatile composition revealed the variability and peculiarity of each sea salt according to its origin. Ocean currents are shown to influence the sea salt volatile composition. Nevertheless, ten compounds were tentatively identified in all sea salts: 6-methyl-5-hepten-2-one, 2,2,6-trimethylcyclohexanone, isophorone, ketoisophorone, β-ionone-5,6-epoxide, dihydroactinidiolide, 6,10,14-trimethyl-2-pentadecanone, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate, 2,4,4-trimethylpentane-1,3-diyl bis(2-methylpropanoate) and 2-ethyl-1-hexanol. Seven of these are carotenoid-derived compounds that can be associated to the typical natural surroundings of ocean hypersaline environment. Thus, this sub-set of ten common compounds is proposed as potential volatile markers of sea salt.

III.B.1. VOLATILE PROFILE OF SEA SALTS

The volatile composition of salts from the Atlantic Ocean, collected at different locations, namely *Île de Ré*, *Aveiro*, *Figueira da Foz*, *Castro Marim*, *Cádiz*, *La Palma* and *Sal* islands, comprised a total of 165 compounds, ranging from 32 (LP salt) to 71 (S salt) compounds per salt sample. These were distributed over several chemical groups: 64 hydrocarbons, 8 aldehydes, 7 esters, 2 haloalkanes, 19 ketones, 2 ethers, 8 alcohols, 30 terpenic compounds, 19 carotenoid derivatives, 3 lactones, 1 furan, 1 sulfide, and 1 pyridine (**Table 3.2** and **Fig. 3.3**). From these, 37 were identified for the first time in sea salt.

The most reliable way to confirm the identification of each compound is based on authentic standard co-injection, which in several cases is economically prohibitive, and often unachievable in the time available for analysis, or because standards are not commercially available. Hence many compounds are tentatively identified based on library match and retention index (RI). The full data matrix (**Table 3.2**) includes a list of all 165 compounds, corresponding retention times in both dimensions, RI obtained through the modulated chromatogram, and the RI reported in the literature for one dimensional GC with a 5%-phenyl-methylpolysiloxane GC column or equivalent. These chromatographic data are crucial for identification purposes. Furthermore, GC×GC is an ideal technique for the analysis of complex mixtures where compounds of similar chemical structure are grouped into distinct patterns in the 2D chromatographic plane providing useful information on their boiling point and polarity (as a non-polar / polar column set was used), and relationships of structured retentions have proved especially useful for compound identification (**Table 3.2**). This unique peculiarity of the GC×GC chromatogram is a powerful tool in the identification step.

GC peak area reproducibility, expressed as relative standard deviation (RSD), varied among sea salts volatile components, ranging from 1 to 163%, which may be explained by the fact that sea salt is a natural heterogeneous product (Silva et al., 2009; Silva et al., 2010b; Silva et al., 2010a).

Table 3.2. Volatile composition of sea salts from several origins (IR, AV, FF, CM, CD, LP, and S) and inland salts (CG and PF).

^1t_R[a]	^2t_R[a]	Compound	RI_{lit}[b]	RI_{calc}[c]	Previously reported[d]	Peak area[e] (× 10-5) and R.S.D. (%) IR	AV	FF	CM	CD	LP	S	CG	PF
		Hydrocarbons												
		Aliphatics												
725	0.95	Undecane	1100	1101	B	1.6 (67)	-	-	-	-	-	-	-	-
845	1.14	Dodecane	1200	1201	A,B	-	-	-	-	-	-	2.21 (14)	-	-
925	1.14	C_{12} isomer	-	1268	-	26.4 (97)	-	-	-	-	-	-	-	-
940	1.17	C_{12} isomer	-	1280	-	7.7 (15)	-	-	-	-	-	-	-	-
955	1.22	C_{12} isomer	-	1293	-	3.4 (13)	-	-	-	-	-	-	-	-
980	1.03	C_{13} isomer	-	1312	-	16.7 (13)	-	-	-	-	-	-	-	-
995	1.05	C_{13} isomer	-	1324	-	6.1 (22)	-	-	-	-	-	-	-	-
1010	1.05	C_{13} isomer	-	1335	-	5.1 (13)	-	-	-	-	-	-	-	-
1050	1.16	2,6,10-Trimethyl-dodecane	-	1366	B	-	-	-	-	-	-	0.65 (28)	-	-
1060	1.06	C_{13} isomer	-	1374	-	1.5 (64)	-	-	-	-	-	-	-	-
1085	1.08	Tetradecane	1400	1397	A,B	-	-	1.46 (9)	-	-	1.66 (13)	-	-	-
1150	1.10	C_{14} isomer	-	1441	-	3.2 (16)	-	-	-	-	-	1.58 (11)	-	-
1160	1.09	C_{14} isomer	-	1449	-	4.1 (8)	1.20 (12)	-	-	-	-	3.18 (5)	-	-
1170	1.10	C_{14} isomer	-	1456	-	3.3 (15)	-	-	-	-	-	2.13 (5)	-	-
1190	1.11	C_{14} isomer	-	1471	-	-	-	-	-	-	-	7.74 (4)	-	-
1205	1.10	C_{14} isomer	-	1482	-	25.7 (11)	-	-	-	-	-	2.15 (7)	-	-
1215	1.14	C_{14} isomer	-	1490	-	8.1 (16)	-	-	-	2.26 (18)	3.24 (7)	-	-	-
1215	1.93	C_{14} isomer	-	1490	-	-	-	-	-	2.10 (27)	-	-	-	-
1235	1.13	Pentadecane	1500	1505	A	6.8 (12)	-	-	-	-	-	2.07 (43)	-	-
1240	1.30	C_{15} isomer	-	1509	-	11.0 (17)	-	-	-	-	-	-	-	-
1265	1.13	C_{15} isomer	-	1528	-	50.9 (14)	-	-	-	-	-	-	-	-
1265	1.34	C_{15} isomer	-	1528	-	21.2 (15)	-	-	-	-	-	7.33 (14)	-	-
1280	1.16	C_{15} isomer	-	1539	-	20.7 (4)	-	-	-	-	-	8.60 (13)	-	-
1290	1.15	C_{15} isomer	-	1547	-	13.7 (26)	-	-	-	-	-	3.70 (25)	-	-
1310	1.12	C_{15} isomer	-	1562	-	6.4 (10)	-	-	-	-	-	2.34 (6)	-	-
1320	1.32	C_{15} isomer	-	1570	-	2.3 (10)	-	-	-	-	-	1.78 (11)	-	-
1330	1.14	C_{15} isomer	-	1578	-	1.5 (20)	-	-	-	-	-	2.12 (13)	-	-
1340	1.37	C_{15} isomer	-	1586	-	2.0 (13)	-	-	-	-	-	-	-	-
1350	1.13	Hexadecane	1600	1593	A	-	-	-	-	-	1.49 (7)	-	-	-
1375	1.16	C_{16} isomer	-	1613	-	-	-	-	-	-	-	8.14 (16)	-	-
1390	1.16	C_{16} isomer	-	1625	-	-	-	-	-	-	-	3.66 (16)	-	-
1405	1.14	C_{16} isomer	-	1637	-	-	-	1.09 (23)	-	2.48 (26)	-	-	-	-
1410	1.38	2,6,10-Trimethyl-pentadecane	1629	1641	B	1.2 (33)	-	-	1.99 (11)	-	-	12.14 (18)	-	-
1420	1.20	C_{16} isomer	-	1649	-	1.4 (8)	-	-	-	-	-	-	-	-
1425	1.38	C_{16} isomer	-	1653	-	1.5 (16)	-	-	-	-	-	10.25 (18)	-	-
1440	1.18	C_{16} isomer	-	1665	-	2.1 (12)	-	-	-	-	-	-	-	-
1450	1.17	C_{16} isomer	-	1673	-	3.6 (24)	-	-	-	-	-	4.79 (19)	-	-
1460	1.45	1-Heptadecene	-	1681	B	-	17.23 (14)	0.88 (21)	-	4.65 (26)	-	-	-	-
1470	1.37	C_{16} isomer	-	1689	-	19.7 (19)	-	-	-	-	-	-	-	-
1475	1.19	Heptadecane	1700	1693	A,B	-	-	-	-	4.57 (25)	3.56 (16)	-	-	-
1500	1.21	C_{17} isomer	-	1710	-	-	-	-	-	1.31 (31)	-	6.35 (22)	-	-
1525	1.18	C_{17} isomer	-	1728	-	33.6 (20)	-	-	-	-	-	14.57 (8)	-	-
1535	1.42	C_{17} isomer	-	1731	-	13.5 (26)	1.17 (44)	-	-	-	-	8.83 (13)	-	-

1550	1.42	C_{17} isomer	-	1744	-	16.3	(22)	-		-		-	1.95	(18)	-		4.87	(18)	-		-	
1555	1.21	C_{17} isomer	-	1748	-	15.4	(11)	-		-		-	-		-		3.88	(11)	-		-	
1575	1.38	C_{17} isomer	-	1758	-	7.9	(40)	-		-		-	-		-		-		-		-	
1585	1.22	C_{17} isomer	-	1768	-	4.9	(21)	-		-		-	-		-		2.22	(19)	-		-	
1630	1.46	Octadecane	1800	1798	-	1.3	(16)	-		-		-	-		-		9.32	(17)	-		-	
1680	1.22	C_{18} isomer	-	1840	-	-		-		-		-	-		-		1.84	(20)	-		-	
1695	1.21	C_{18} isomer	-	1853	-	-		-		-		-	-		-		4.91	(17)	-		-	
1705	1.22	C_{18} isomer	-	1862	-	-		-		-		-	-		-		2.83	(81)	-		-	
1715	1.20	C_{18} isomer	-	1871	-	-		-		-		-	-		-		6.69	(13)	-		-	
1725	1.23	C_{18} isomer	-	1879	-	2.1	(41)	-		-		-	-		-		4.07	(29)	-		-	
1735	1.43	C_{18} isomer	-	1888	-	-		-		-		-	-		-		6.74	(22)	-		-	
1780	1.44	C_{19} isomer	-	1929	-	4.1	(53)	-		-		-	-		-		6.28	(40)	-		-	
		Aromatics																	-			
1405	1.73	(1-Butylheptyl)-benzene	1631	1637	B	-		0.47	(46)	0.73	(17)	0.48	(13)	-	0.38	(5)	-		-		-	
1415	1.59	(1-Propyloctyl)-benzene	1643	1645	B	-		0.19	(49)	0.35	(30)	0.20	(15)	-	-		-		-		-	
1525	1.50	(1-Pentylheptyl)-benzene	1711	1728	-	-		0.97	(67)	-		0.96	(11)	-	0.59	(11)	-		-		-	
1530	1.45	(1-Butyloctyl)-benzene	1716	1731	B	-		-		0.75	(53)	-		-	-		-		-		0.71	(66)
1545	1.62	(1-Propylnonyl)-benzene	1725	1741	B	-		-		0.16	(15)	-		-	-		-		-		-	
1580	1.76	(1-Ethyldecyl)-benzene	1790	1764	-	-		-		-		0.17	(15)	-	-		-		-		-	
1645	1.77	(1-Hexylheptyl)-benzene	-	1810	-	-		0.86	(20)	-		-		-	0.15	(31)	-		-		-	
1655	1.77	(1-Butylnonyl)-benzene	1812	1819	-	-		0.26	(89)	-		0.24	(5)	-	-		-		-		-	
1675	1.81	(1-Propyldecyl)-benzene	1843	1836	-	-		-		-		0.13	(16)	-	-		-		-		-	
		Subtotal (GC peak area)				378.3	(20)	22.35	(10)	5.42	(21)	4.17	(1)	19.32	(22)	11.06	(10)	169.94	(8)	-		-	
		Subtotal (%)				77.13	(5)	4.66	(6)	3.78	(15)	1.80	(0)	3.75	(19)	15.51	(10)	72.10	(8)	-		-	
		Aldehydes																					
		Aliphatics																					
525	1.28	Heptanal	916	906	B	-		-		-		3.09	(44)	-	-		-		-		-	
640	1.34	Octanal	1012	1015	B	-		-		1.11	(105)	13.69[f]	(5)	-	-		1.81	(19)	34.10	(32)	1.45	(102)
745	1.44	Nonanal	1127	1111	B	0.67	(56)	1.21	(26)	4.30	(94)	36.50	(1)	-	2.23	(18)	5.20	(27)	65.06	(14)	3.41	(7)
860	1.55	Decanal	1216	1214	B	-		1.18	(31)	6.64	(79)	41.13	(17)	-	1.40	(33)	5.53	(42)	50.14	(27)	2.26	(28)
985	1.58	Undecanal	1316	1317	B	-		-		-		3.55	(29)	-	-		-		-		-	
1115	1.66	Dodecanal	1411	1416	B	-		-		0.74	(39)	2.86	(30)	-	-		0.17	(99)	9.58	(19)	0.51	(87)
1665	2.08	5,9,13-Trimethyl-4,8,12-tetradecatrienal	-	1828	B	-		-		0.41	(12)	-		-	-		-		-		-	
		Aromatics																					
615	2.42	Benzaldehyde	986	993	B	-		-		-		2.81	(15)	-	2.22	(16)	-		-		-	
		Subtotal (GC peak area)				0.67	(56)	2.39	(26)	13.20	(81)	103.63	(7)	-	5.85	(20)	12.71	(33)	-		-	
		Subtotal (%)				0.14	(55)	0.50	(26)	8.34	(71)	44.81	(5)	-	8.19	(19)	5.31	(24)	-		-	
		Esters																					
		Aliphatics																					
740	1.39	Isopentyl 3-methylbutanoate	1101	1106	-	-		-		-		-		-	-		0.10[f]	(123)	-		-	
1045	2.74	1-Hydroxy-2,4,4-trimethylpentan-3-yl 2-methylpropanoate	-	1364	B	0.14	(144)	1.56	(11)	5.83	(10)	0.86	(38)	1.30	(44)	-		-		-		-	
1075	**2.50**	**3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate[g]**	**1381**	**1387**	**B**	**3.89**	**(42)**	**11.13**	**(15)**	**11.48**	**(29)**	**9.55**	**(23)**	**1.19**	**(54)**	**3.32**	**(15)**	**1.81**	**(112)**	-		-	
1340	**1.89**	**2,4,4-Trimethylpentane-1,3-diyl bis(2-methylpropanoate)**	**1605**	**1586**	**B**	**1.04**	**(63)**	**2.42**	**(35)**	**7.72**	**(95)**	**1.29**	**(68)**	**1.76**	**(27)**	**0.71**	**(40)**	**0.40**	**(134)**	-		**4.47**	**(130)**
1875	1.87	Isopropyl palmitate	2011	2011	B	-		-		-		0.52	(30)	-	-		-		-		-	
		Aromatics																					
1705	3.12	Dibutyl phthalate	1897	1864	B	-		-		28.87	(143)	-		-	-		-		-		-	
1830	3.05	Bis(2-methoxyethyl) phthalate	1990	1976	-	-		-		1.79	(107)	-		-	-		-		-		-	

		Subtotal (GC peak area)				5.07	(47)	15.11	(13)	55.68	(84)	12.21	(29)	4.26	(39)	4.03	(19)	2.31	(116)	-		-	
		Subtotal (%)				1.01	(33)	3.18	(20)	35.88	(61)	5.27	(27)	0.82	(36)	5.64	(18)	0.92	(110)	-		-	
		Haloalkanes																					
700	1.27	1-Chloro-octane	1044	1065	B	0.70	(31)	0.43	(40)	-		0.46	(20)	-		0.57	(18)	-		-		-	
810	1.25	1-Chloro-nonane	1159	1170	B	-		-		0.29	(49)	0.52	(51)	-		-		-		3.25	(27)	-	
		Subtotal (GC peak area)				0.70	(31)	0.43	(40)	0.29	(49)	0.98	(36)	-		0.57	(18)	-		-		-	
		Subtotal (%)				0.15	(48)	0.09	(38)	0.19	(33)	0.42	(35)	-		0.81	(19)	-		-		-	
		Ketones																					
		Aliphatics																					
670	1.48	1-(2,2-Dimethylcyclopentyl)-ethanone	-	1042	B	-		1.99	(25)	-		-		-		-		-		-		-	
690	2.21	2,3-Dimethyl-2-cyclopenten-1-one	-	1061	B	-		1.91	(5)	-		-		1.02	(9)	-		-		-		-	
710	1.69	3,6,6-Trimethylcyclohex-2-enone	-	1075	B	5.65	(11)	8.41	(16)	1.72	(10)			3.57	(5)	1.26	(6)	0.37	(22)	-		-	
715	2.33	3-Methyl-2-cyclohexen-1-one	-	1084	B	-		1.82	(4)	-		-		1.72	(13)	-		-		0.53	(26)	-	
730	1.43	2-Nonanone	1083	1097	B	0.60	(31)	1.12	(19)	-		-		0.79	(10)	0.19	(7)	-		5.00	(33)	-	
760	2.27	3,5-Dimethyl-2-cyclohexen-1-one	-	1125	-	-		0.24	(3)	-		-		-		-		-		-		-	
810	2.12	6,6-Dimethylbicyclo[3.1.1]heptan-2-one	1142	1166	B	-		0.42	(19)	-		-		-		-		-		-		-	
830	1.47	3,3,4,4-Tetramethyl-2-pentanone	-	1188	B	-		-		-		-		0.47	(13)	-		-		-		-	
830	1.87	6,6-Dimethyl-2-methylene-bicyclo[2.2.1] heptan-3-one	1114	1188	-	-		1.26	(118)	-		-		-		-		-		-		-	
845	1.50	2-Decanone	1196	1198	B	-		0.66	(33)	-		-		-		-		-		3.75	(29)	-	
890	2.32	1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-6-one	-	1239	B	-		0.62	(27)	-		-		-		-		-		-		-	
965	1.73	2-Undecanone	1294	1301	B	-		1.02	(26)	0.24	(20)	-		0.90	(21)	-		-		-		-	
980	1.02	1-Aza-2-cycloheptanone	1266	1312	-	-		-		-		-		26.70	(82)	-		-		-		-	
1010	3.01	2-(2-Methylpropylidene)-cycloheptanone	-	1337	B	-		-		-		-		0.94	(2)	-		-		-		-	
1095	3.26	3-Acetyl-2,4,4-trimethylcyclohex-2-en-1-one	1422	1402	-	-		-		-		-		0.30	(29)	-		-		-		-	
1130	2.49	6-Methyl-6-(5-methylfuran-2-yl)heotan-2-one	1479	1428	B	1.15	(15)	1.31	(10)	0.49	(17)	-		1.74	(7)	0.23	(29)	-		-		-	
1195	1.82	2,6-bis(1,1-Dimethylethyl)-2,5-cyclohexadiene-1,4-dione	1468	1479	-	-		-		-		-		-		-		1.25	(74)	-		1.75	(16)
1215	2.08	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	1490	1490	B	-		-		-		-		0.39	(26)	-		0.38	(33)	-		-	
		Aromatic																					
725	2.59	Acetophenone	1065	1093	B	-		-		-		-		1.36	(26)	-		-		3.79	(27)	-	
		Subtotal (GC peak area)				7.41	(7)	20.77	(7)	2.45	(8)	-		39.89	(55)	1.68	(1)	2.00	(49)	-		-	
		Subtotal (%)				1.55	(21)	4.41	(23)	1.80	(39)	-		7.64	(49)	2.35	(1)	0.82	(40)	-		-	
		Ethers																					
565	1.49	1-Methoxy-4-methyl-benzene	-	940	B	-		1.11	(51)	-		-		-		-		-		-		-	
1435	1.48	Dioctyl ether	-	1657	B	0.51	(83)	0.54	(25)	0.43[f]	(117)	-		-		-		-		-		-	
		Subtotal (GC peak area)				0.51	(83)	1.66	(43)	0.43	(117)	-		-		-		-		-		-	
		Subtotal (%)				0.11	(82)	0.36	(53)	0.26	(121)	-		-		-		-		-		-	
		Alcohols																					
		Aliphatics																					
665	**1.97**	**2-Ethyl-1-hexanol**	**1029**	**1038**	**A,B**	**3.09**	**(88)**	**8.60**	**(10)**	**1.84**	**(47)**	**9.13**	**(37)**	**2.04**	**(7)**	**2.16**	**(12)**	**9.24**	**(68)**	**1206.55**	**(17)**	**94.52**	**(31)**
735	2.35	1-Undecyn-4-ol	-	1102	B	-		-		-		0.18	(3)	-		-		-		0.70[f]	(112)	-	
765	2.22	2,6-Dimethylcyclohexanol	1112	1129	-	-		-		-		1.03	(3)	62.08	(7)	-		-		-		-	
1080	1.36	1-Undecanol	1370	1386	B	-		-		-		0.42	(5)	-		-		0.36	(23)	-		-	
1110	2.09	2-Dodecanol	1387	1413	B	-		-		-		-		0.73	(15)	-		-		-		-	
1340	1.41	1-Tridecanol	1583	1586	B	-		0.33	(19)	-		0.39	(10)	-		-		-		-		-	
1415	1.29	3,7,11-Trimethyl-1-dodecanol	-	1642	B	-		-		-		-		1.44	(23)	-		-		-		-	
1465	2.37	1-Tetradecanol	1673	1686	B	-		-		-		0.47	(22)	1.45	(29)	-		-		-		-	

		Subtotal (GC peak area)				3.09	(88)	8.93	(9)	1.84	(47)	11.63	(29)	67.75	(6)	2.16	(12)	9.61	(66)	-		-	
		Subtotal (%)				0.61	(76)	1.88	(19)	1.26	(31)	5.05	(30)	13.23	(8)	3.03	(11)	3.96	(58)	-		-	
		Terpenic compounds																					
625	1.21	2,3-Dehydro-1,8-cineole	994	1001	-	-		-		-		0.54	(22)	-		-		-		-		-	
665	1.27	Cymene	1038	1042	A,B	-		-		-		0.37	(8)	-		-		-		1.72	(64)	-	
675	1.23	1,8-Cineole	1032	1042	A,B	-		12.37	(128)	0.21	(101)	-		-		-		-		-		-	
710	1.83	Dihydromyrcenol	1072	1079	B	0.33	(13)	-		-		0.45	(46)	3.80	(22)	0.23	(9)	-		-		-	
715	1.76	Linalool oxide (isomer)	1087	1083	-	-		0.81	(27)	-		-		-		-		0.68	(31)	-		-	
730	1.88	Linalool oxide (isomer)	1087	1097	-	-		-		-		-		1.26	(2)	-		0.57	(54)	-		-	
740	1.89	Linalool	1098	1106	B	-		-		-		-		0.42	(58)	-		-		1.08	(26)	-	
810	1.97	Dihydro terpineol	1152	1170	-	-		-		-		-		-		-		0.09	(5)	-		-	
815	1.69	Camphor	1143	1174	B	0.34	(28)	0.71	(22)	-		-		0.59	(10)	0.33	(8)	0.31	(3)	10.00	(19)	-	
820	1.48	Menthone (isomer)	1154	1179	-	0.32	(42)	-		-		-		-		0.33	(17)	0.36	(3)	14.72	(27)	-	
830	3.10	Pulegone (isomer)	1237	1189	-	-		0.29	(19)	-		-		-		-		-		-		-	
835	2.27	Umbellulone	1171	1193	-	-		-		-		-		0.41	(14)	-		-		-		-	
845	2.01	Menthan-1-ol	-	1202	-	-		-		-		0.36	(41)	-		-		-		1.89	(36)	-	
845	2.22	Isomenthol	1178	1197	B	0.18	(153)	-		-		-		-		-		-		-		-	
865	2.23	α-Terpineol	1206	1219	-	-		0.79	(9)	-		1.39	(12)	0.25	(52)	-		0.73	(16)	1.29	(16)	-	
890	2.49	Verbenone	1204	1235	B	-		1.01	(14)	-		-		-		-		0.70	(17)	-		-	
905	2.50	Eucarvone	1248	1252	B	-		0.46	(14)	-		-		-		-		-		-		-	
930	2.45	Carvone	1242	1281	B	-		-		-		2.40	(13)	-		-		-		-		-	
1040	1.88	Neryl acetate	1365	1359	-	-		-		-		-		0.45	(12)	-		-		-		-	
1245	1.74	Muurolene	1506	1513	-	-		-		-		-		-		-		1.43	(30)	1.02	(43)	-	
1270	1.78	Cadinene	1525	1532	-	-		-		-		-		-		-		7.39	(20)	-		-	
1280	1.76	Calamenene	1524	1540	-	-		-		-		-		-		-		5.08	(10)	1.25	(17)	-	
1315	2.12	Calacorene	1546	1567	-	-		-		-		-		-		-		6.44	(5)	-		-	
1405	2.27	Ledol	1609	1637	-	-		-		-		-		-		-		1.73	(60)	-		-	
1410	2.34	C_{15} (*m/z* 41, 43, 55, 119, 105)	-	1642	-	-		-		-		-		-		-		1.40	(58)	-		-	
1430	2.33	C_{15} (*m/z* 41, 119, 43, 105, 55)	-	1658	-	-		-		-		-		-		-		1.98	(85)	-		-	
1445	2.62	Cadinol	1651	1670	-	-		-		-		-		-		-		0.61	(67)	-		-	
1450	2.67	Muurolol	1661	1674	-	-		-		-		-		-		-		1.17	(81)	-		-	
1490	2.53	Cadalene	1674	1708	-	-		-		-		-		-		-		1.46	(38)	1.70	(12)	-	
1605	1.18	2,6,10,14-Tetramethyl-hexadecane	-	1781	B	36.36	(14)	12.27	(14)	6.34	(12)	29.77	(19)	5.37	(15)	3.25	(13)	-		22.40	(14)	2.55	(30)
		Subtotal (GC peak area)				37.53	(14)	28.71	(60)	6.55	(15)	35.28	(14)	12.55	(13)	4.13	(10)	32.14	(19)	-		-	
		Subtotal (%)				7.94	(30)	5.77	(49)	4.66	(24)	15.26	(14)	2.45	(13)	5.79	(11)	13.52	(10)	-		-	
		Carotenoid derivatives																					
580	1.43	6-Methyl-2-heptanone	953	958	A, B	-		15.93	(7)	17.71	(111)	39.38	(15)	36.75	(21)	27.39	(1)	-		-		-	
600	1.64	2-Methyl-1-hepten-6-one	966	983	-	1.67	(12)	-		-		-		-		-		-		-		-	
620	**1.49**	**6-Methyl-5-hepten-2-one**	**1003**	**997**	**A,B**	**5.73**	**(8)**	**7.72**	**(7)**	**3.95**	**(5)**	**4.11**	**(15)**	**0.87**	**(37)**	**5.57**	**(10)**	**4.66**	**(36)**	**4.97**	**(64)**	**-**	
645	1.61	2,4,4-Trimethylcyclopentanone	-	1020	B	-		8.57	(9)	-		-		4.02	(21)	-		-		-		-	
680	**1.51**	**2,2,6-Trimethylcyclohexanone**	**1036**	**1051**	**A,B,C**	**15.83**	**(21)**	**12.10**	**(9)**	**6.79**	**(19)**	**0.24**	**(6)**	**5.42**	**(35)**	**1.46**	**(4)**	**0.23**	**(12)**	**-**		**-**	
730	1.80	3,4,4-Trimethyl-2-cyclohexen-1-one	1097	1097	B	3.35	(4)	10.01	(9)	1.47	(29)	-		-		0.36	(13)	-		-		-	
740	2.01	3,4,4-Trimethyl-2-cyclopenten-1-one	-	1106	-	3.35	(4)	-		-		-		-		-		-		0.84	(16)	-	
785	**2.24**	**Isophorone (isomer)**	**1121**	**1147**	**B,C**	**0.27**	**(12)**	**5.11**	**(12)**	**1.13**	**(21)**	**0.22**	**(4)**	**1.17**	**(8)**	**0.33**	**(1)**	**0.54**	**(29)**	**-**		**-**	
810	**2.46**	**Ketoisophorone**	**1169**	**1171**	**B**	**2.44**	**(9)**	**50.70**	**(1)**	**11.05**	**(27)**	**2.89**	**(6)**	**20.04**	**(10)**	**1.74**	**(2)**	**0.11**	**(79)**	**0.48**	**(20)**	**-**	
840	3.02	2,2,6-Trimethyl-1,4-cyclohexanedione	1190	1197	B,C	0.09	(150)	9.83	(9)	6.00	(33)	1.25	(18)	6.02	(13)	-		-		-		-	
895	2.05	β-Cyclocitral	1223	1243	A,B,C	3.23	(6)	3.90	(10)	1.19	(10)	-		2.46	(9)	0.56	(7)	-		-		-	
1100	1.61	6,10-Dimethyl-2-undecanone	1410	1405	A,B	-		-		-		8.84	(18)	-		-		-		-		-	
1145	2.31	α-Ionone	1426	1439	A,B,C	3.08	(12)	21.69	(7)	2.37	(5)	-		3.46	(9)	0.48	(9)	-		-		-	
1160	2.21	Dihydro-β-ionone	1424	1450	B	-		4.89	(10)	-		-		-		-		-		-		-	

1220	2.46	β-Ionone	1485	1494	A,B	1.09	(33)	3.00	(12)	0.62	(6)	-		2.43	(7)	-		-		-		
1230	**2.64**	**β-Ionone-5,6-epoxide**	**1463**	**1498**	**B**	**6.77**	**(26)**	**24.17**	**(19)**	**5.07**	**(23)**	**0.26**	**(18)**	**76.04**	**(7)**	**1.66**	**(18)**	**0.52**	**(102)**	**-**		**-**
1325	**4.34**	**Dihydroactinidiolide (isomer)**	**1539**	**1580**	**A,B,C**	**3.41**	**(35)**	**198.96**	**(27)**	**1.92**	**(49)**	**1.02**	**(52)**	**187.78**	**(5)**	**1.81**	**(12)**	**0.02**	**(74)**	**-**		**-**
1330	4.11	Dihydroactinidiolide (isomer)	1539	1588	B	-		-		0.56[f]	(90)	-		-		-		-		-		-
1665	**1.76**	**6,10,14-Trimethyl-2-pentadecanone**	**1843**	**1832**	**A,B**	**0.86**	**(42)**	**2.23**	**(31)**	**1.88**	**(16)**	**3.75**	**(55)**	**5.22**	**(36)**	**0.50**	**(38)**	**0.32**	**(45)**	**-**		**-**
		Subtotal (GC peak area)				51.16	(8)	378.83	(15)	61.70	(43)	61.97	(10)	351.67	(1)	41.85	(2)	6.40	(12)	-		-
		Subtotal (%)				10.60	(12)	78.57	(4)	43.72	(40)	26.83	(11)	68.66	(6)	58.68	(3)	2.75	(22)	-		-
		Lactones																				
1010	2.25	2,2,4-Trimethyl-5-(2,2-dimethylpropyl)-3(2H)-furanone	-	1336	-	-		-		-		-		-		-		0.72	(45)	-		-
1215	4.23	7a-Methyl-3-methylenehexahydrobenzofuran-2-one	-	1492	B	-		2.44	(14)	-		-		2.18	(20)	-		-		-		-
1285	3.73	Tetrahydroactinidiolide	-	1545	-	3.00	(23)	-		-		-		5.95	(81)	-		-		-		-
		Subtotal (GC peak area)				3.00	(23)	2.44	(14)	-		-		8.13	(57)	-		0.72	(45)	-		-
		Subtotal (%)				0.63	(31)	0.51	(2)	-		-		1.62	(59)	-		0.30	(37)	-		-
		Others																				
620	1.17	2-Pentylfuran	996	997	B	0.63	(7)	0.33	(48)	0.20[f]	(108)	1.29	(14)	-		-		0.71	(42)	-	0.58	(59)
620	1.79	Dimethyl trisulfide	972	997	B	-		-		-		-		5.45	(163)	-		-		-		-
630	1.66	2,4,6-Trimethyl-pyridine	1014	1006	-	-		-		-		-		4.16	(30)	-		-		-		-
		Subtotal (GC peak area)				0.63	(7)	0.33	(48)	0.20	(108)	1.29	(14)	9.62	(83)	-		0.71	(42)	-		-
		Subtotal (%)				0.13	(12)	0.07	(44)	0.12	(112)	0.56	(12)	1.82	(78)	-		0.71	(42)	-		-
		Total				488.05	(16)	481.96	(15)	147.75	(31)	231.15	(2)	513.19	(5)	71.34	(1)	236.54	(10)			
		Number of identified compounds				69		55		40		44		52		32		71				

Sea Salts Code: *Île de Ré* – IR; Aveiro – AV; *Figueira da Foz* – FF; *Castro Marim* – CM; *Cádiz* – CD; *La Palma* island – LP; *Sal* island – S. Inland Salts Code: *Coarse Gold* – CG, *Pink Flakes* – PF. For inland salts was only included data about compounds common with sea salts

[a] Retention times in seconds (s) for first (^1t_R) and second (^2t_R) dimensions

[b] RI: retention index reported in the literature for 5% phenyl-dimethyl polysilphenylene-siloxane GC column or equivalents (Georgilopoulos & Gallois, 1987; Adams, 1995; Elmore et al., 1999; Högnadóttir & Rouseff, 2003; Valim et al., 2003; Leffingwell & Alford, 2005; Pino, 2007; Zeng et al., 2007; Hoet et al., 2010; Babushok & Zenkevich, 2009; M^{c}Ginitie & Harynuk, 2012; Vasta et al., 2012)

[c] RI: retention index obtained through the modulated chromatogram

[d] A – Silva et al., 2009; B – Silva et al., 2010; C – Donadio et al., 2011; D – Serrano et al., 2011

[e] Mean of three replicates

[f] The compound was detected in two replicates

[g] Compounds highlighted in bold are common to all sea salts

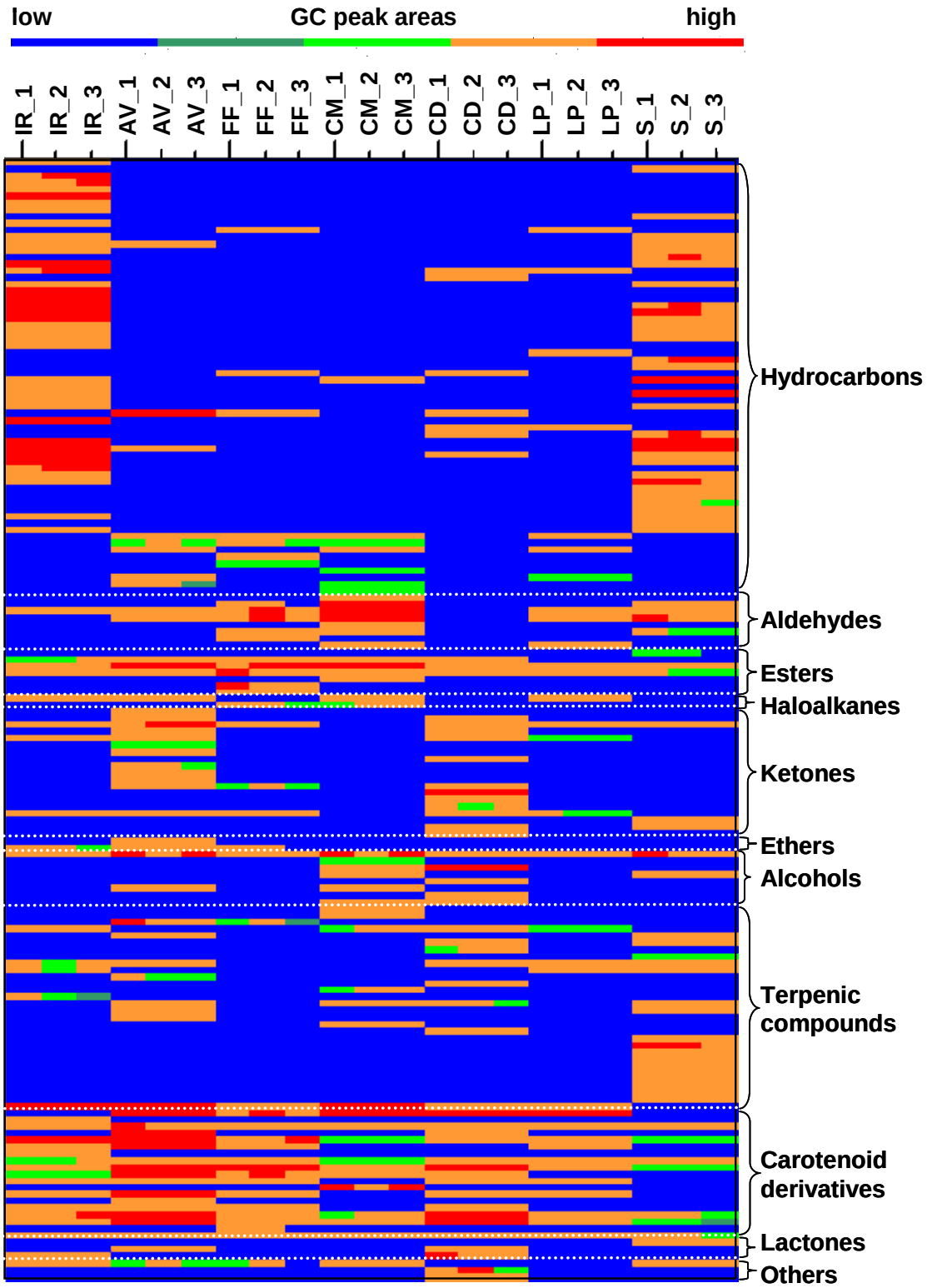

Figure 3.3. Heatmap representation of GC×GC peak areas from sea salt volatile components. *Île de Ré* – **IR**; *Aveiro* – **AV**; *Figueira da Foz* – **FF**; *Castro Marim* – **CM**; *Cádiz* – **CD**; *La Palma island* – **LP**; *Sal island* – **S**. Areas are normalized by applying a logarithm function.

In order to facilitate the analysis of the full data set concerning the volatile profile of the sea salts under study, a heatmap representation and a hierarchical cluster analysis (HCA) were performed. The heatmap (**Fig. 3.3**) shows a graphical representation of the in-depth data present in **Table 3.2** and allows a rapid visual evaluation of the similarities and differences between samples, whereas the dendrogram (**Fig. 3.4a**) built from the HCA, is an exploratory tool designed to reveal natural groupings, and groups sea salts according to their similarities.

Fig. 3.3 heatmap shows that S and IR samples exhibited the greater number of compounds, while a fewer number of volatile compounds was detected for LP. This was mainly related to the major presence of hydrocarbons in IR and S, and also with the presence of the sesquiterpenoids (included in the group of terpenic compounds in **Table 3.2**), only identified in S. The heatmap shows that the carotenoid derivatives profile was very similar among all the samples under study.

The occurrence of ketones, as well perceptible in the heatmap, was greater for AV and CD. Among the major chemical groups identified, FF presented fewer terpenic compounds, and S of carotenoid derivatives. For IR and S, the hydrocarbons was the chemical group showing the largest GC peak area, whereas for CM it was the aldehydes, and for the other salts the carotenoid derivatives (**Table 3.2** and **Fig. 3.3**).

Fig. 3.4a presents the dendrogram built from the hierarchical cluster analysis of the GC peak area of all the 165 volatile compounds. The vertical axis of the dendrograms measures the similarity between samples: lower height corresponds to higher similarity between the samples. **Fig. 3.4a** shows that the replicates of each sea salt exhibit less variability, i.e. the intravariability among replicates was lower than intervariability among the 7 samples. Although the volatile composition of each sea salt seems to be unique, which may be related to its particular saltpan environment, similarities among all sea salts can be observed. Three principal clusters may be observed (**Fig. 3.4a**): S salt, IR salt, and a cluster with the remaining salts (CM, LP, FF, CD, and AV). The oceans are dynamic systems, with currents a paramount factor to be considered, and they may promote a genetic connectivity according to the marine organisms among regions provided by the same current. The Atlantic Ocean currents are shown in **Fig. 2.1** (**Chapter II**), and an analogy can be suggested between the three clusters previously defined (**Fig. 3.4a**) and the North Equatorial current, the Canary current, and North Atlantic Drift current (**Fig. 2.1**).

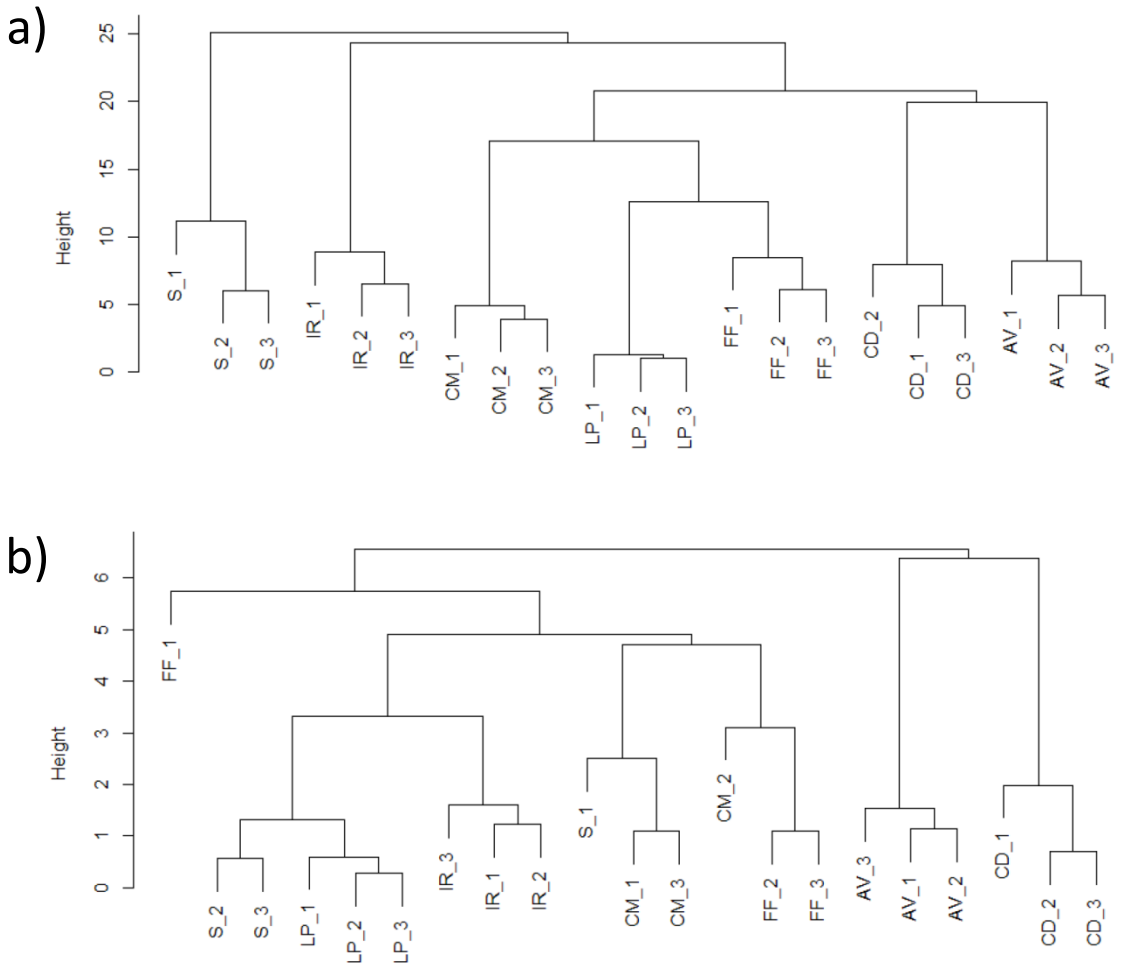

Figure 3.4. Dendrograms from GC peak areas of sea salt volatile compounds. (a) All volatile compounds (165) detected in Atlantic Ocean sea salts under study. (b) Sub-set of ten potential volatile markers common to all Atlantic Ocean salts. (*Île de Ré* – **IR**; *Aveiro* - **AV**; *Figueira da Foz* – **FF**; *Castro Marim* – **CM**; *Cádiz* – **CD**; *La Palma* island – **LP**; *Sal island* - **S**)

S, situated in a more southerly location, seems to be influenced by the North Equatorial current, coming from east towards northwest, IR, located in a more northerly geographical area, seems to be influence by the North Atlantic drift coming from west toward northeast, and the remaining saltpans, with a central position nearest to the Strait of Gibraltar, appear to be influenced by a descending current starting as a North Atlantic Drift and finishing as

the Canary current. These different currents seem to influence the volatile composition of sea salt.

III.B.2. POTENTIAL VOLATILE MARKERS OF SEA SALT

From the 165 volatile components of sea salt, a sub-set of ten compounds was identified in all samples under study (**Fig. 3.5**): seven carotenoid derivatives, i.e. 6-methyl-5-hepten-2-one (6MHO) (**1**), 2,2,6-trimethylcyclohexanone (TCH) (**2**), 3,5,5-trimethyl-2-cyclohexenone (isophorone) (**3**), 2,6,6-trimethyl-2-cyclohexene-1,4-dione (ketoisophorone) (**4**), 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-3-buten-2-one (β-ionon-5,6-epoxide) (**5**), dihydroactinidiolide (DHA) (**6**), and 6,10,14-trimethyl-2-pentadecanone (TPD) (**7**), two esters, i.e. 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate (3HPP) (**8**), and 2,4,4-trimethylpentane-1,3-diyl bis(2-methylpropanoate) (TMPP) (**9**), and the alcohol 2-ethyl-1-hexanol (2EH) (**10**). Thus, although the volatile composition of sea salts is related with the surrounding environment where these are collected, there is a base composition characteristic of all sea salts.

To explore possible natural groupings (or clusters) within sea salts using this sub-data set, HCA was performed (**Fig. 3.4b**). This dendrogram showed a higher similarity between samples compared to the dendrogram comprising all the volatile compounds (**Fig. 3.4a**). It was also verified that sea salt replicates of FF, S, and CM were not closely grouped. Based on the HCA of the sub-set of ten common compounds, it was not possible to group salts by saltpan, suggesting that they characterise the sea salt itself rather than their origin/environment. Thus, this sub-set of compounds was defined as sea salt potential volatile markers.

To confirm this hypothesis, the presence of these 10 volatile compounds was checked in several sea salts and non-sea salts (inland salts) analysed by HS-SPME/GC×GC–ToFMS. Concerning the sea salts, this sub-set was detected in five sea salts from a different year (2010), namely from Aveiro, Figueira da Foz, Castro Marim, Île de Ré, and Guérande (also situated in the Western Coast of France). The occurrence of this sub-set was also checked in sea salts previously analysed (Silva et al., 2010a), comprising two saltpans of Aveiro, harvested in 2007, and the ten compounds

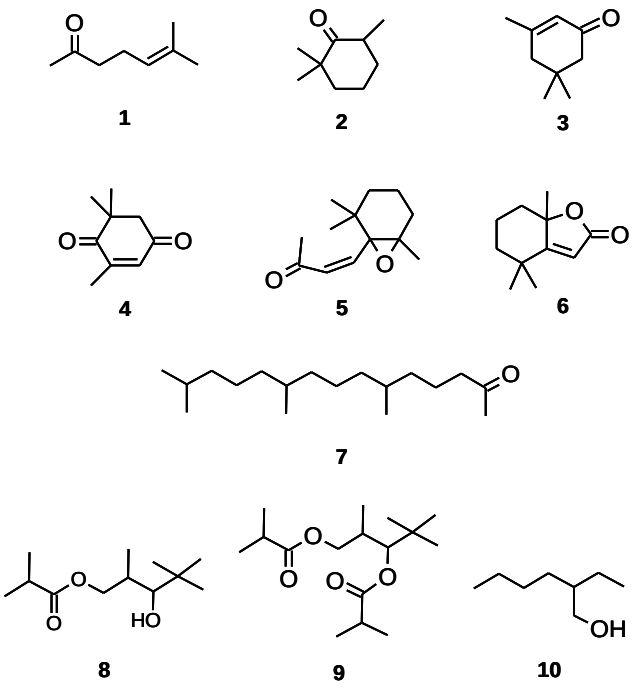

Figure 3.5. Chemical structures of the ten tentatively identified compounds common to all Atlantic Ocean salts. (**1**) 6-methyl-5-hepten-2-one; (**2**) 2,2,6-trimethylcyclohexanone; (**3**) isophorone; (**4**) ketoisophorone; (**5**) β-ionone-5,6-epoxide; (**6**) dihydroactinidiolide; (**7**) 6,10,14-trimethyl-2-pentadecanone; (**8**) 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate; (**9**) 2,4,4-trimethylpentane-1,3-diyl bis(2-methylpropanoate); (**10**) 2-ethyl-1-hexanol.

were detected in both sea salts. Moreover, this study also comprised the analysis of sea salts stored for two and three years, and only 3HPP, 2EH, 6MHO, ketoisophorone, DHA, and TPD were detected. These results can be explained by the longer storage time, which contributes for the loss of volatile compounds, including these potential markers (Silva et al., 2010a).

To verify if potential markers of sea salt might also occur in inland salt produced in saltpans away from the coast, where groundwater sourced salt is released from natural brine aquifers, two inland salts from Australia (CG and PF) were analysed by the same HS-SPME/GC×GC–ToFMS methodology. Among the sub-set of ten compounds, only 4 potential volatile markers were identified, and only 2-ethyl-1-hexanol was detected in both inland salts. 6-Methyl-5-hepten-2-one and ketoisophorone were detected only in CG

sample, and 2,4,4-trimethyl-1-(2-methylpropanoyloxy)pentan-3-yl] 2-methylpropanoate in PF (**Table 3.2**). These results corroborate the proposed hypothesis about the existence of potential markers of sea salt.

In addition, it may be pointed out that only 28 compounds were detected in common with the sea salts under study, 24 for CG, and 10 for PF (**Table 3.2**). The volatile profile of inland salts produced far from the sea differs from that of sea salts, taking also into account the greater number of volatiles only detected in the inland salts under study (data not shown). Although unlikely, an ancient sea source for the inland salt might still contains some of its volatile compounds possibly preserved in its crystalline structure.

An in-depth understanding of the origin of volatiles from sea salt is crucial to interpret the surrounding environment of saltpans, taking into consideration the microbial communities, plants, and algae.. Although useful, this information is very scarce and may be unavailable in the literature for the locations under study. However, as far as possible, a general overview about these data was undertaken (**Fig. 3.6**), with particular emphasis on the 10 potential volatile markers. Considering the potential sources of volatile compounds previously reported for sea salt (Silva *et al.*, 2009; Silva *et al.*, 2010b; Silva *et al.*, 2010a; Donadio *et al.*, 2011; Serrano *et al.*, 2011), namely, algae, halophyte, bacteria, and anthropogenic activity, it was reported the potential contribution of these sources for the several chemical groups detected (**Fig. 3.6**) using references that may support this information (Evans, 1994; Elenkov *et al.*, 1995; Krock & Wilkins, 1996; Beauchêne *et al.*, 2000; Grossi & Raphel, 2003; Gao *et al.*, 2004; Kamenarska *et al.*, 2006; Nalli *et al.*, 2006; Shibata *et al.*, 2006; El Hattab *et al.*, 2007; Erakin & Güven, 2008; Gressler *et al.*, 2009; Amorri *et al.*, 2011; Sun *et al.*, 2012). Algae proved to be a potential source for the majority of the chemical groups identified. The occurrence of these potential sources depends on the surrounding environment of saltpans and contributes to the existence of different sea salt volatile profiles (**Fig. 3.3**).

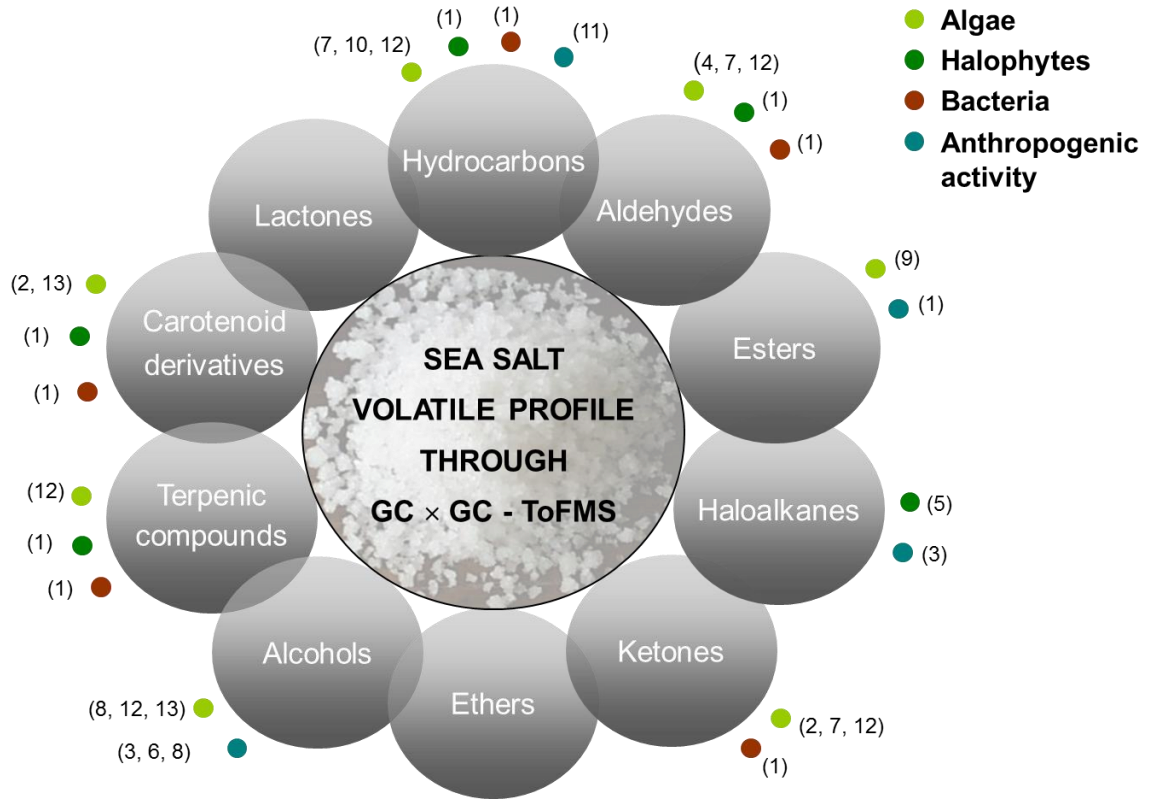

Figure 3.6. Potential sources of the chemical groups of sea salt volatile compounds.

(**1** - Evans, 1994; **2** - Elenkov *et al.*, 1995; **3** - Krock & Wilkins, 1996; **4** - Beauchêne *et al.*, 2000; **5** - Grossi & Raphel, 2003; **6** - Gao *et al.*, 2004; **7** - Kamenarska *et al.*, 2006; **8** - Nalli *et al.*, 2006; **9** - Shibata *et al.*, 2006; **10** - El Hattab *et al.*, 2007; **11** - Erakin & Güven, 2008; **12** - Gressler *et al.*, 2009; **13** - Amorri *et al.*, 2011; **14** - Sun *et al.*, 2012)

Seven from the 10 sea salt potential volatile markers are carotenoid derivatives. These may arise from carotenoid degradation, such as from the natural pigment β-carotene (Winterhalter & Rouseff, 2002) present in plants and algae. For instance, halophilic microalga *Dunaliella salina* accumulates large amounts of β-carotene (Lamers *et al.*, 2008). The compound 6MHO was already reported as a component of the halophytes *Salicornia europaea rubra* and *Puccinellia nuttalliana* (Evans, 1994), and marine brown algae *Fucus serratus* and *Hormophysa cuneiformis* (Beauchêne *et al.*, 2000; El Hattab *et al.*, 2007). TDP was also reported to be related to the former brown algae (El Hattab *et al.*, 2007). Carotenoid derivatives 6MHO, TCH, and TPD were previously identified as volatile components of the marine green alga *Capsosiphon fulvescens* (Sun *et al.*, 2012). 6MHO, along with isophorone and ketoisophorone, were previously identified in a shore-dwelling cyanobacterial mat community from the hypersaline Wells lake, in Canada

(Evans, 1994). β-Ionone-5,6-epoxide and TPD were found in the marine red algae *Bostrychia radicans* and *Bostrychia tenella* growing on the rocky shore (Oliveira *et al.*, 2009). DHA has already been identified in several kinds of marine algae (Gressler *et al.*, 2009), such as the green alga *Cladophora vagabunda* (Elenkov *et al.*, 1995), and numerous red algae (Kamenarska *et al.*, 2006). TPD has been found as a component of *Zostera marina* shoots (Kawasaki *et al.*, 1998), an aquatic plant of marine environments also known as eelgrass, and in the marine green alga *Ulva pertusa* (Gressler *et al.*, 2009). The majority of the above algae exist in the Atlantic Ocean (http://www.algaebase.org/).

Considering the non-carotenoid derivatives, it may be pointed out that 2EH was previously identified in marine brown algae *Fucus serratus* (Beauchêne *et al.*, 2000), and 3HPP was also detected as a minor compound in the secretions of marine brown algae *Eisenia bicyclis* and *Ecklonia kurome* (Shibata *et al.*, 2006). 3HPP, and also 6MHO, were routinely identified in the coastal atmosphere (oceanic and continental air masses) of Mace Head in Ireland, with 6MHO and 2EH the major oxygenated compounds of these environments (Sartin *et al.*, 2001). In this study 6MHO was considered to be ubiquitous and of biogenic origin while 2EH and 3HPP were of unclear origin. 2EH has been reported as having a non-natural source since it is used in the production of plasticizers for polyvinyl chloride (PVC) resins and as an intermediate in the manufacture of inks, paper, rubber, resins, surfactants, and lubricants (Staples, 2001). It can also be produced by bacteria and fungi from degradation of plasticizers (Nalli *et al.*, 2006). No marine or hypersaline origins have been found for the non-carotenoid derivative TMPP. However, this compound was already identified in natural sources, such as *Scapania verrucosa* Heeg, and its endophytic fungus *Chaetomium fusiforme* (Guo *et al.*, 2008). Consequently, the non-carotenoid derivatives present in all the salts analysed may result from the integration of compounds from anthropogenic activity on the metabolism of marine organisms.

III.B.3. CONCLUDING REMARKS

Analysis of the volatile composition of sea salts from *Île de Ré, Aveiro, Figueira da Foz, Castro Marim, Cádiz,* and from *La Palma* and *Sal* islands, produced in 2007, enabled the detection of 165 compounds distributed over the chemical groups of hydrocarbons,

aldehydes, esters, haloalkanes, ketones, ethers, alcohols, terpenic compounds, carotenoid derivatives, and lactones. In spite of the particular volatile profile of each salt under study modulated by several factors, namely by ocean currents, and saltpan surrounding environment, it was possible to identify a sub-set of ten compounds common to all, namely 6-methyl-5-hepten-2-one, 2,2,6-trimethylcyclohexanone, isophorone, ketoisophorone, β-ionone-5,6-epoxide, dihydroactinidiolide, 6,10,14-trimethyl-2-pentadecanone, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate, 2,4,4-trimethylpentane-1,3-diyl bis(2-methylpropanoate), and 2-ethyl-1-hexanol. These compounds have their origin in the environmental elements such as plants, algae, bacteria of marine and/or other hypersaline environments. These potential volatile markers seem to characterise sea salt, independently from saltpan, geographic origin and harvest. Volatile markers of sea salt could play an important role in the differentiation of this natural product from industrial salt, and so add to the value of sea salt as a distinct and desirable product. A more extensive study should be conducted including samples collected across the world's oceans. On the other hand, although representing minor components, the volatile compounds of sea salt may be seen as flavour compounds with a potential contribution in the organoleptic and chemical properties of the foodstuffs where sea salt is added, leading to potential sea salt taste modulators.

III.C. HIGHLY SULFATED POLYSACCHARIDES FROM SEA SALT: ISOLATION AND CHARACTERISATION

A dialysis-based methodology was developed to isolate, for the first time, polymeric material from sea salt in amounts that allow its characterisation. The content of polymeric material isolated from 16 Atlantic sea salts with different geographical origins and year of production had a median of 144 mg per kg of salt. Polymeric material from sea salt was mainly composed by highly sulfated polysaccharides, and comprised also protein and traces of nucleic acids. Polysaccharides were rich in uronic acids (21 mol%), glucose (18 mol%), galactose (15 mol%), and fucose (13 mol%). The 4-linked-Gal*p* was the major residue of polysaccharides from sea salt whereas fucose and galactose were the main sugar residues, highly substituted by sulfate. One gram of polymeric material from sea salt was composed by 461 mg of sulfated polysaccharides, which corresponds to 66 mg/kg of sea salt. According to the glycosidic linkages identified these polysaccharides seem to arise mainly from marine algae.

III.C.1. ISOLATION OF POLYMERIC MATERIAL FROM SEA SALT

The first approach to obtain the polymeric material (PM) from sea salt was the dialysis of a saturated solution (36% (w/w) at 25ºC). With this purpose, an aqueous solution (200 mL) containing 75 g of sea salt was dialysed, allowing to obtain about 10 mg of PM. However, in this process, the amount of sea salt dialysed per experiment was very low. Based on the fact that the solids do not contribute to the osmotic pressure of the solutions, the sea salt was directly inserted into the dialysis bag filling it completely. Then, water was added until all the empty spaces were filled, allowing the complete removal of the air. The bag was then tied and immersed in water (**Fig. 2.11**). Using this procedure, it was possible to observe the slow dissolution of the salt in the water and the diffusion of the salt solution into the dialysate. For each salt, 60 g of sea salt were dialysed in each bag having a volume of 80 mL. In a total, for each one of the 16 samples used, 480 g of each were dialysed. All samples used were from the Atlantic Ocean, from distinct locations: Portugal (Aveiro, Figueira da Foz, and Castro Marim), Spain main land (Cádiz), France (Île de Ré), Canarias Islands (La Palma Island), and Cape Verde (Sal Island). For some of these origins, sea salt samples with different years of production were analysed, as referred in the section 2.1 (Chapter II).

The amount of PM ranged from 47 to 348 mg per kg of sea salt dry weight (**Table 3.3**). The freeze-dried PM from all samples presented a fluffy aspect with a pearl colour (**Fig. 2.11**). Considering all sea salt samples analysed, it was possible to estimate that one kilogram of Atlantic Ocean sea salt contains a median of 144 mg of PM, e.g 0.014% (w/w).

III.C.2. MID-INFRARED PROFILE OF POLYMERIC MATERIAL FROM SEA SALT

Fig. 3.7 shows the overlapped mid-infrared spectra in the 4000 – 500 cm^{-1} wavenumber region of the polymeric material isolated from all sea salt samples. Given the similar spectrum profile, it was possible to identify typical bands regarding the polymeric material composition from sea salt. The spectra showed high absorbance at wavenumbers that can be attributed to polysaccharides: 3360 cm^{-1} O-H stretching, 2935 cm^{-1} saturated C-

H stretching, 1200 – 850 cm^{-1} stretching of C–O in C–O–H bonds of carbohydrates (McCann *et al.*, 1992; Coimbra *et al.*, 1998; Černá *et al.*, 2003; Qian *et al.*, 2009), and proteins: 1640 cm^{-1} C=O stretching (Amide I), 1550 cm^{-1} C-N stretching and N-H bending (Amide II) (McCann *et al.*, 1992; Kong & Yu, 2007). Two other strong bands can be attributed to the presence of sulfate: 1380 cm^{-1} ester sulfate, 1250 cm^{-1} S=O stretching (Karmakar *et al.*, 2009; Souza *et al.*, 2012). Analysis of the acquired spectra profiles and comparison with several other references for which spectral similarities were found reveals the presence of sulfated polysaccharides in the polymeric material from sea salt, possibly, carrageenans, agarans, ulvans and/or fucoidans (Liao *et al.*, 1996; Marais & Joseleau, 2001; Černá *et al.*, 2003; Pourjavadi *et al.*, 2004; Karmakar *et al.*, 2009; Alves *et al.*, 2012; Guerrero *et al.*, 2014).

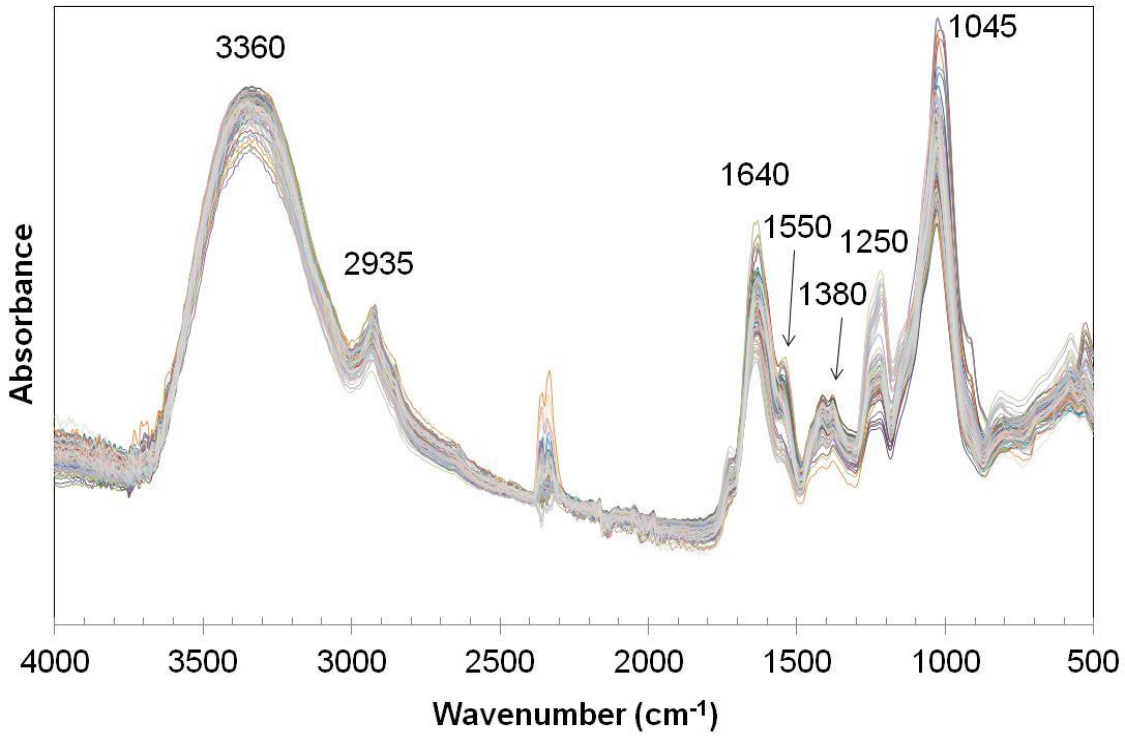

Figure 3.7. MIR spectra overlap (4000 – 500 cm^{-1}) of the polymeric material isolated from different sea salts.

The occurrence of sulfated polysaccharides in sea salt could have origin in the marine environment which is abundant in organisms rich in sulfated polysaccharides such

as seaweeds and marine invertebrates (Mestechkina & Shcherbukhin, 2010). However, sulfated polysaccharides were also reported in microorganisms such as microalgae, namely *Dunaliella salina* (Dai *et al.*, 2010; Mishra *et al.*, 2011), and halophilic bacteria (Béjar *et al.*, 1998; Arias *et al.*, 2003), known to colonize saline soils, thus representing other potential sources.

III.C.3. THERMAL ANALYSIS OF THE POLYMERIC MATERIAL FROM SEA SALT

Thermal degradation of the polymeric material from sea salt was studied by thermogravimetric analysis (TGA) in the temperature range from room temperature to 600ºC at a rate of 10ºC per minute. The overlap of TGA and derivative thermogravimetric (DTG) profiles of polymeric material samples from all sea salt analysed is represented in **Fig. 3.8**. Since thermograms of all the samples analysed follow a similar behaviour, a typical profile for the polymeric material from sea salt weight loss was observed with the increase of temperature. A first small loss of weight occurs near 100ºC due to dehydration which accounts for the moisture content (Biswas & Staff, 2001; Abdou *et al.*, 2008). Near 240ºC the degradation of protein amino acid residues can give rise to a second loss of weight (Biswas *et al.*, 2001; Yi *et al.*, 2004). The higher loss of mass, occurring near 360ºC, can be attributed to polysaccharide degradation (Sagheer *et al.*, 2009; Cerqueira *et al.*, 2011). This high weight loss can be followed by the formation of volatile degradation products (Zawadzki & Kaczmarek, 2010). For most samples, 20 to 50% of their weight remained at 600ºC. This could be related to the presence of sulfate coming from sulfated polysaccharides, which is not lost at temperatures below 600ºC (Silva *et al.*, 2004; Anastasakis *et al.*, 2011). The presence of water in the polymeric material from sea salt (~ 3%) confirms that this is a hygroscopic material, since it was stored in a desiccator with P_2O_5 (s) until analysis, and even in these conditions it absorbed water. Based on the thermograms obtained, it can be inferred that the polymeric material from sea salt was mainly composed by polysaccharides, possibly sulfated, and by a small amount of protein.

In order to obtain more information about the structure of these polysaccharides, sugar and glycosidic-linkage analysis were performed.

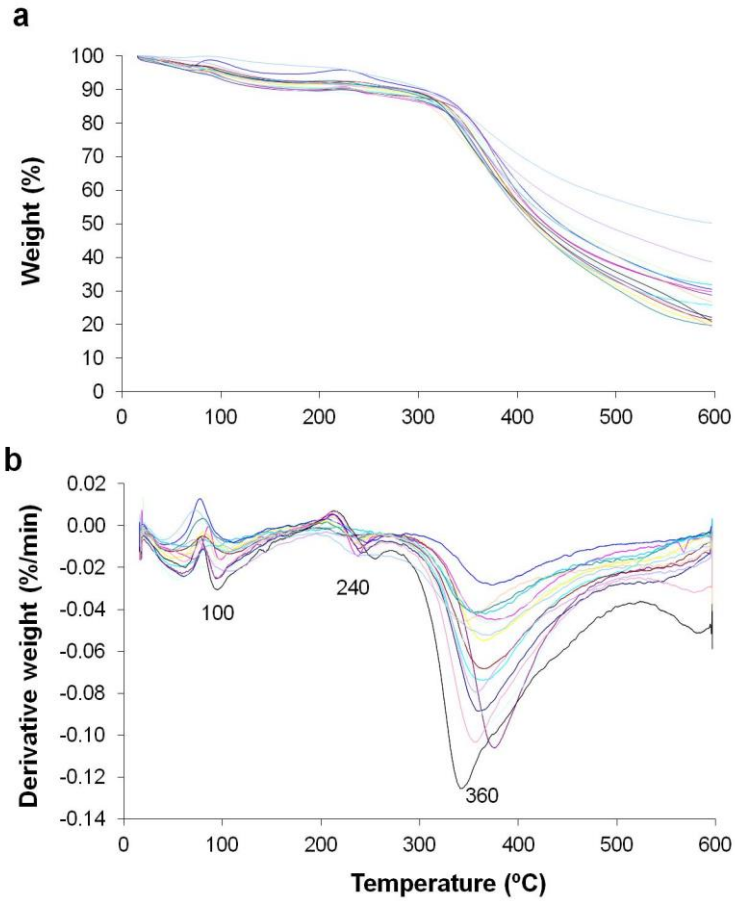

Figure 3.8. Overlap of (a) TGA and (b) DTG profiles of the polymeric material isolated from different sea salts.

III.C.4. CHARACTERISATION OF POLYSACCHARIDES FROM SEA SALT

The sugar composition of the PM isolated from the different sea salt samples is shown in **Fig. 3.9** and **Table 3.3**. The total sugar content of the PM of the 16 samples analysed had a median of 335 mg/g, varying from 275 to 471 mg per g of PM. For the majority of the samples, the main sugars identified were uronic acids (20 mol%), glucose (17 mol%), galactose (15 mol%), and fucose (12 mol%). Xylose (9 mol%), mannose (9 mol%), rhamnose (8 mol%), arabinose (5 mol%), deoxyribose (3 mol%), and ribose (1%) were also identified.

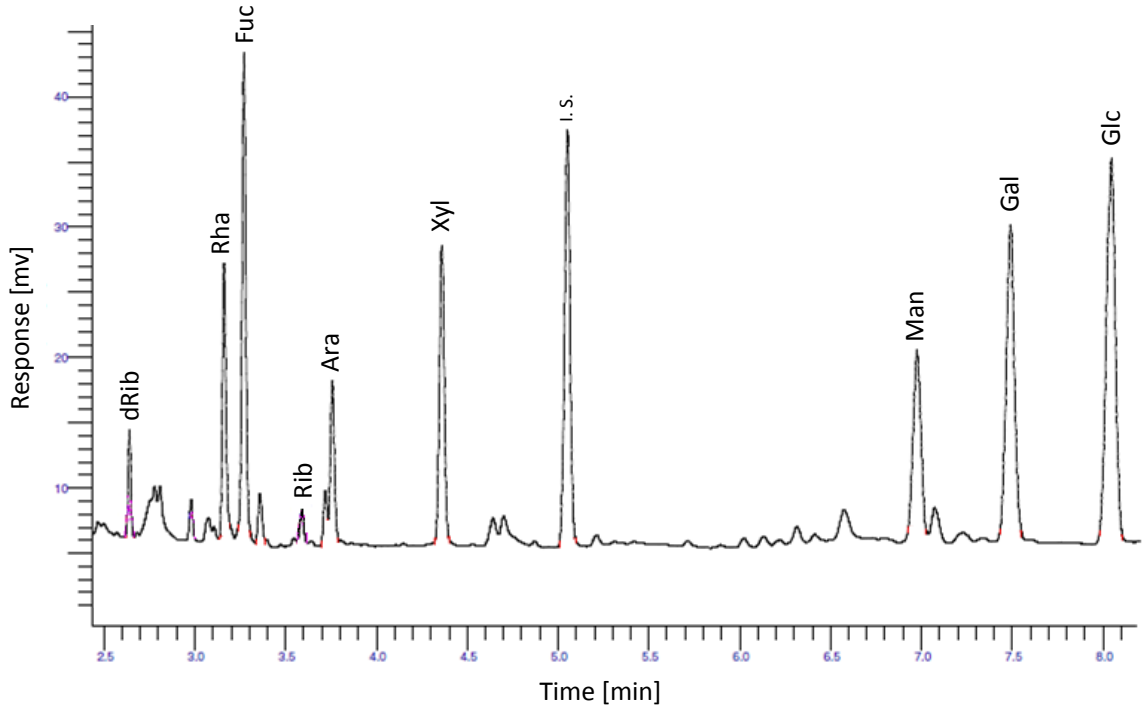

Figure 3.9. GC-FID chromatogram of the sugar composition of PM from sea salt FF04. (I.S.- Internal standard)

The presence of deoxyribose and ribose indicates that sea salt contains minor amounts of DNA and RNA, respectively. The amount of these two sugars, although present in the samples, due to their possible non-polysaccharide origin, was not considered for the quantification of polysaccharides from sea salt. Sulfate content ranged from 78 to 246 mg per g of PM, representing a median of 133 mg/g. This justifies the sulfate bands detected by mid-infrared spectroscopy and the material resistant at 600ºC, and can be attributed to the presence of sulfated polysaccharides, representing the sulfate contenta median of 45 mol%. Therefore, PM content on total polysaccharides was 461 mg/g, e.g. it ranged from 364 to 699 mg per g. This means that in one kilogram of sea salt it is possible to find a median of 66 mg of sulfated polysaccharides. The presence of uronic acids and sulfate in the polysaccharide composition of PM from sea salt turns these polysaccharides more soluble in water preventing its earlier precipitation in the saltpan. With a wide range of possible origins, it was expected that the sugar fraction existing in sea salt should comprise more than one type of polysaccharide.

Table 3.3. Sugar and sulfate composition of the polymeric material isolated from different sea salts.

Samples	Yield[a]	Sugars (mol%)										Total Sugars[b]	Sulfate[b]	Total Polysaccharides[b]
	(mg/kg)	dRib[c]	Rha	Fuc	Rib[c]	Ara	Xyl	Man	Gal	Glc	UA	(mg/g)	(mg/g)	(mg/g)
PJ04	123	4	8	11	1	4	9	11	14	14	25	306	133	429
PJ05	171	4	8	12	1	4	8	10	15	16	22	326	128	443
PJ07	167	3	8	14	1	5	8	8	15	14	24	361	154	505
18C04	140	3	7	12	1	9	9	8	16	16	19	313	166	469
18C05	178	3	8	16	1	6	8	8	15	17	18	304	86	379
18C07	148	4	8	12	1	5	8	8	14	15	24	318	137	442
GCa07	116	3	8	16	1	4	8	9	15	16	21	323	166	480
FF04	96	3	7	12	1	5	10	8	15	20	19	341	132	463
FF05	104	2	7	13	1	5	12	9	15	20	17	352	78	421
FF07	98	3	8	14	1	5	10	9	15	18	18	341	128	460
CM07	47	5	5	6	1	5	8	10	15	20	26	275	101	364
CD07	348	3	4	12	1	5	11	9	12	19	25	471	238	693
IR07	160	2	8	19	1	6	9	8	14	15	17	374	132	498
LP07	230	3	10	10	1	3	11	9	19	18	17	444	167	599
LP09	336	2	10	9	0	3	9	8	22	20	15	462	246	699
S07	46	4	2	7	1	4	8	12	17	23	21	329	96	412
SEA SALT	157[d] (55)[e]	3 (28)	7 (29)	12 (26)	1 (23)	5 (30)	9 (13)	9 (14)	15 (16)	18 (15)	21 (17)	353 (16)	143 (33)	485 (20)
	144[f]	3	8	12	1	5	9	9	15	17	20	335	133	461

[a] Expressed in mg of polymeric material per kg of sea salt dry weight (4-10% of water)
[b] Expressed in mg per g of polymeric material
[c] As deoxyribose and ribose are DNA and RNA components, respectively, they were not considered for the calculation of total polysaccharides from sea salt
[d] Average
[e] Relative standard deviation (%)
[f] Median

In order to infer what were the types of polysaccharides present in PM from sea salt, glycosidic linkages were analysed by methylation of the polysaccharides. The methylation procedure used included a first methylation and dialysis step, followed by a remethylation and a second dialysis, assuring that all OH groups have been methylated. **Fig. 3.10** shows an example of a GC-qMS chromatogram of partially methylated alditol acetates of polysaccharides from sea salt. Methylation analysis (**Table 3.4**) showed that polysaccharides coming from different sea salt samples revealed a similar chemical structure. Due to the low amount of PM, methylation analysis was not performed for sample S07. The major residue identified in the polysaccharides from sea salt was 4-linked Gal*p*, representing a median of 9.3 mol%. This was already identified in carrageenans of red algae along with T-Gal*p*, 3-Gal*p*, 3,4-substituted Gal*p*, 2,4-substituted Gal*p*, 4,6-substituted Gal*p*, 3,6-substituted Gal*p*, 3,4,6-substituted Gal*p* and 2,3,4,6-substituted Gal*p*

(Kolender & Matulewicz, 2002; Chattopadhyay *et al.*, 2007; Navarro *et al.*, 2007; Wang *et al.*, 2007; Bondu *et al.*, 2010; Jiao *et al.*, 2011; Salehi *et al.*, 2011; Yang *et al.*, 2011). According to each residue, sulfate can be located in different positions. In 3,4-substituted Gal*p* this can occupy C-3 or C-4 (Kolender *et al.*, 2002; Bilan *et al.*, 2007), in 2,4-substituted Gal*p* the C-2 (Wang *et al.*, 2007), and for 4,6-substituted Gal*p* and 3,6-substituted Gal*p* this can be located in C-6 (Villanueva *et al.*, 2010; Jiao *et al.*, 2011). Carrageenan can present 3,6-anhydro-galactose in its structure, however with the procedure used in this study it was not possible to detect it (Stevenson & Furneaux, 1991). Another major residue identified, 4-linked Xyl*p* (median of 3.8 mol%), was already found in a sulfated heteroglycuronan of green alga *Enteromorpha compressa* (Ray, 2006). Other residues found in sea salt, namely T-Rha*p*, 2-linked Rha*p*, 3-linked Rha*p*, 3,4-substituted Rha*p*, 2,3,4-substituted Rha*p*, 2,3,4-substituted Xyl*p*, 6-linked Gal*p* and T-Glc*p*, were also identified in this green alga polysaccharide. Sulfate was found in C-3 of the 3,4-substituted Rha*p* (Ray, 2006). The residue 2,3,4-substituted Fuc*p* together with all the remaining fucose residues identified in sea salt were already found in fucoidans from brown algae (Marais *et al.*, 2001; Bilan *et al.*, 2007; Li *et al.*, 2008; Karmakar *et al.*, 2009). Sulfate was found in C-4 of 3,4-substituted Fuc*p*, in C-2 of 2,3-substituted Fuc*p*, and simultaneously in C-2 and C-3 or C-2 and C-4 of 2,3,4-substituted Fuc*p*, forming a disulfated residue. The residues 2,6-substituted

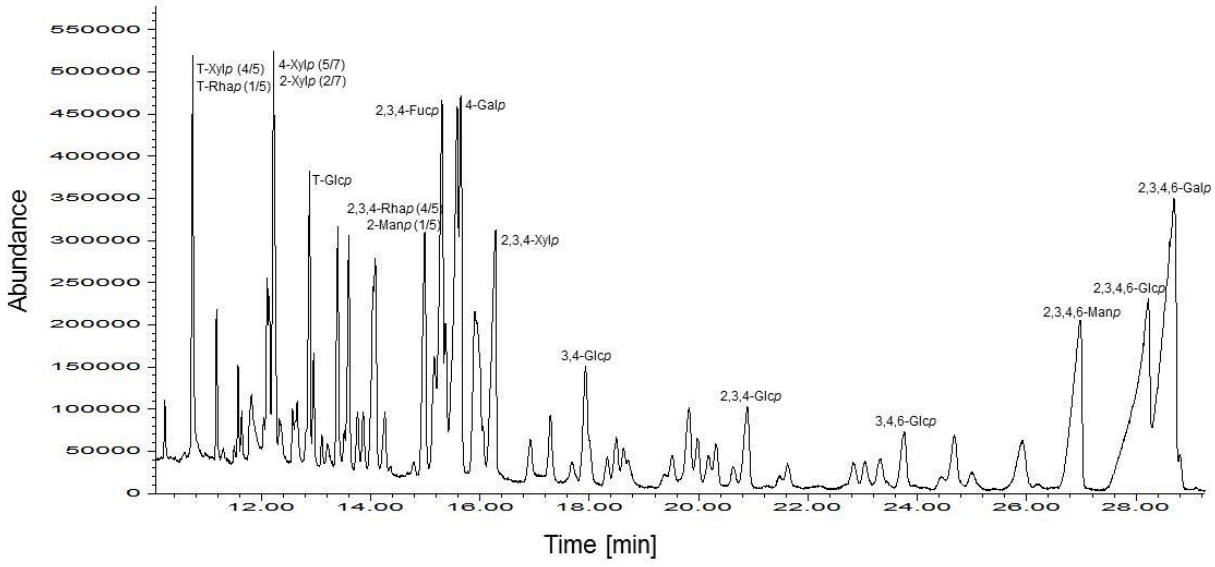

Figure 3.10. GC-qMS chromatogram of the glycosidic linkages composition of polysaccharides from sea salt 18C04.

Table 3.4. Methylation analysis (mol %) of the polymeric material isolated from different sea salt samples.

Methylated derivatives[a]	Sugar derivatives	PJ04	PJ05	PJ07	18C04	18C05	18C07	GCa07	FF04	FF05	FF07	CM07	CD07	IR07	LP07	LP09	Average	Median
2,3,4-Me_3 Rha*p*	T-Rha*p*	1.6	1.4	2.7	0.7	1.9	1.2	2.5	1.3	1.3	0.3	0.3	0.5	1.0	0.5	0.3	1.2 (65)[b]	1.2
3,4-Me_2 Rha*p*	2-Rha*p*	2.6	1.5	2.6	0.9	2.3	2.6	1.2	2.1	1.7	1.8	1.3	1.7	2.5	1.7	1.4	1.9 (29)	1.7
2,4-Me_2 Rha*p*	3-Rha*p*	1.2	0.5	0.6	0.3	0.6	0.7	0.6	0.6	0.5	0.4	tr	0.8	0.7	0.3	tr	0.6 (39)	0.6
2,3-Me_2 Rha*p*	4-Rha*p*	tr	tr	0.3	0.2	0.4	0.3	0.4	0.3	0.4	0.4	tr	0.9	0.5	0.3	tr	0.4 (43)	0.4
2-Me Rha*p*	3,4-Rha*p*	0.4	0.2	0.3	0.3	0.4	tr	0.4	0.4	0.3	0.3	tr	tr	0.4	0.3	0.4	0.3 (20)	0.3
4-Me Rha*p*	2,3-Rha*p*	0.6	0.2	0.3	0.2	0.3	0.5	0.4	0.1	0.2	0.1	0.3	0.3	0.4	0.4	0.4	0.3 (44)	0.3
3-Me Rha*p*	2,4-Rha*p*	0.5	0.6	0.5	0.6	0.6	0.7	0.7	0.7	0.5	0.5	0.4	0.4	0.5	1.3	1.5	0.7 (47)	0.6
Rha*p*	2,3,4-Rha*p*	1.9	3.3	1.6	2.4	1.0	1.4	0.8	1.1	0.8	0.6	1.3	0.6	0.5	2.0	3.2	1.5 (60)	1.3
2,3,4-Me_3 Fuc*p*	T-Fuc*p*	2.4	1.3	3.1	0.7	2.0	1.3	2.9	1.4	2.6	0.7	0.7	0.8	1.6	0.4	0.4	1.5 (62)	1.3
2,4-Me_2 Fuc*p*	3-Fuc*p*	0.4	0.4	0.5	0.4	0.9	0.4	0.8	0.7	0.7	0.5	1.6	0.6	1.1	0.3	0.4	0.7 (53)	0.5
3,4-Me_2 Fuc*p*	2-Fuc*p*	0.7	0.5	0.8	0.5	1.1	0.8	1.0	0.7	0.8	1.1	2.1	1.4	1.0	0.4	0.6	0.9 (46)	0.8
2-Me Fuc*p*	3,4-Fuc*p*	1.9	1.9	1.9	1.9	3.6	2.4	3.8	3.3	2.7	2.4	1.3	3.5	4.7	1.5	1.8	2.6 (38)	2.4
4-Me Fuc*p*	2,3-Fuc*p*	0.4	0.6	0.6	0.6	1.1	0.7	0.9	0.6	0.5	0.5	0.4	0.8	0.9	0.5	0.7	0.7 (30)	0.6
Fuc*p*	2,3,4-Fuc*p*	3.3	7.1	3.7	5.0	3.5	3.4	2.7	2.6	3.7	1.2	2.9	2.5	3.7	2.4	4.1	3.5 (39)	3.4
2,3,5-Me_3 Ara*f*	T-Ara*f*	0.8	0.9	2.6	0.3	0.6	0.3	0.8	0.6	0.8	-	-	0.1	0.5	-	-	0.6 (116)	0.5
3,5-Me_2 Ara*f*	2-Ara*f*	0.1	0.2	0.4	0.6	0.5	0.4	0.3	0.2	0.3	tr	-	-	0.4	-	-	0.2 (84)	0.2
2,5-Me_2 Ara*f*	3-Ara*f*	0.2	0.1	0.3	0.6	0.4	tr	0.2	0.2	0.4	tr	-	-	0.2	-	-	0.2 (91)	0.2
2,3-Me_2 Ara*f*	5-Ara*f*	0.8	0.6	0.9	0.6	1.3	0.9	0.7	1.2	1.7	1.2	0.4	1.3	1.4	0.5	0.3	0.9 (45)	0.9
2,4,5-Me_3 Ara*p*	3-Ara*p*	1.3	1.1	1.8	1.0	1.8	1.9	1.7	2.1	1.3	1.8	1.1	3.9	2.0	1.2	1.3	1.7 (43)	1.7
2-Me Ara*f*	3,5-Ara*f*	0.3	0.3	0.4	0.6	0.7	0.5	0.7	0.7	1.0	0.6	0.6	1.1	0.8	0.6	0.7	0.7 (34)	0.6
4,5-Me_2 Ara*f*	2,3-Ara*p*	0.4	0.2	0.3	0.4	0.5	0.4	0.5	0.7	0.7	0.5	0.3	0.6	0.5	0.2	0.3	0.5 (33)	0.4
3,5-Me_2Ara*p*	2,4-Ara*p*	0.9	0.9	1.1	1.6	1.1	0.9	0.7	1.2	0.8	1.0	1.0	0.6	1.1	1.0	0.8	1.0 (24)	1.0
5-Me Ara*p*	2,3,4-Ara*p*	2.0	3.9	tr	6.7	tr	1.8	-	-	-	-	-	-	-	tr	1.3	1.3 (160)	0.0
2,3,4-Me_3 Xyl*p*	T-Xyl*p*	5.0	4.1	6.9	2.6	5.8	3.5	6.4	5.1	6.1	1.6	1.5	2.8	4.5	1.9	1.1	3.9 (49)	4.1
2,4-Me_2 Xyl*p*	3-Xyl*p*	0.9	0.9	0.9	0.9	1.5	1.1	0.8	2.0	2.3	1.3	0.4	2.6	1.8	1.9	1.4	1.4 (46)	1.3
2,3-Me_2 Xyl*p*	4-Xyl*p*	3.0	2.3	3.8	2.3	4.8	3.8	5.6	5.4	5.3	4.1	2.0	3.5	5.3	3.1	2.3	3.8 (33)	3.8
3,4-Me_2 Xyl*p*	2-Xyl*p*	1.8	1.3	1.7	0.9	1.8	1.4	2.1	1.7	2.1	1.6	1.1	2.2	2.2	1.6	1.1	1.6 (26)	1.7
2-Me Xyl*p*	3,4-Xyl*p*	1.3	0.8	1.0	1.5	1.3	1.2	1.2	1.7	1.4	1.4	0.9	2.2	1.6	1.9	1.6	1.4 (27)	1.4
Xyl*p*	2,3,4-Xyl*p*	3.7	5.8	3.6	4.8	1.4	2.6	0.7	2.2	1.6	0.9	3.3	1.7	1.0	4.1	5.3	2.8 (59)	2.6
2,3,4,6-Me_4 Man*p*	T-Man*p*	1.3	1.0	1.5	0.7	1.6	1.4	1.8	1.3	1.4	1.2	1.2	1.4	1.6	1.2	1.0	1.3 (21)	1.3
3,4,6-Me_3 Man*p*	2-Man*p*	1.5	0.8	0.9	0.6	1.2	1.2	1.5	1.4	1.5	1.4	1.2	1.5	1.7	1.7	1.5	1.3 (25)	1.4
2,3,6-Me_3 Man*p*	4-Man*p*	1.5	0.9	1.5	1.3	2.0	2.0	2.3	1.9	1.8	2.7	2.6	1.5	2.1	2.0	1.5	1.8 (26)	1.9
2,3,4-Me_3 Man*p*	6-Man*p*	1.2	0.5	0.6	0.4	0.6	0.8	0.9	0.8	0.7	0.9	0.9	1.0	0.7	0.9	0.6	0.8 (27)	0.8
4,6-Me_2 Man*p*	2,3-Man*p*	0.7	0.4	0.4	0.4	0.6	0.8	0.6	0.7	0.5	0.7	0.9	0.7	0.8	0.4	0.3	0.6 (31)	0.6
3,6-Me_2 Man*p*	2,4-Man*p*	0.2	0.2	0.2	0.3	0.5	0.6	0.4	0.5	0.4	0.5	0.4	0.3	0.5	0.4	0.3	0.4 (33)	0.4
2,3-Me_2 Man*p*	4,6-Man*p*	0.6	0.4	0.5	0.6	1.0	1.1	1.0	0.9	0.5	1.1	0.6	0.4	0.6	1.2	0.7	0.8 (36)	0.6
3,4-Me_2 Man*p*	2,6-Man*p*	0.4	0.3	0.3	0.5	0.6	0.6	0.4	0.6	0.8	0.7	0.4	0.7	0.5	0.8	0.5	0.5 (30)	0.5
6-Me Man*p*	2,3,4-Man*p*	1.5	1.1	0.5	0.7	0.6	0.6	0.8	0.7	0.5	0.8	1.0	1.3	0.7	0.4	0.5	0.8 (41)	0.7
2,4-Me_2 Man*p*	3,6-Man*p*	0.2	0.2	0.2	0.4	0.3	0.3	0.4	0.3	0.2	0.4	0.2	0.3	0.3	0.4	0.3	0.3 (27)	0.3
2-Me Man*p*	3,4,6-Man*p*	0.3	0.2	0.1	0.6	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.5	0.5	0.3 (49)	0.2
4-Me Man*p*	2,3,6-Man*p*	0.7	0.5	0.3	0.6	0.4	0.5	0.5	0.4	0.4	0.4	0.7	0.7	0.6	0.5	0.5	0.5 (24)	0.5

Methylated derivatives[a]	Sugar derivatives	PJ04	PJ05	PJ07	18C04	18C05	18C07	GCa07	FF04	FF05	FF07	CM07	CD07	IR07	LP07	LP09	Average	Median
3-Me Man*p*	2,4,6-Man*p*	0.5	0.4	0.3	0.6	0.4	0.5	0.4	0.5	0.5	0.4	0.5	0.5	0.5	0.5	0.4	0.5 (16)	0.5
Man*p*	2,3,4,6- Man*p*	4.6	6.9	2.6	5.3	1.2	2.7	0.7	1.4	1.0	0.5	4.1	1.2	0.4	3.1	0.5	2.4 (84)	1.4
2,3,4,6-Me_4 Gal*p*	T-Gal*p*	2.9	2.7	3.6	1.8	5.9	3.3	5.8	3.5	3.1	2.2	2.3	2.2	5.1	2.2	1.8	3.2 (42)	2.9
2,3,6-Me_3 Gal*p*	4-Gal*p*	5.4	4.8	13.1	3.2	7.7	10.6	10.1	9.3	10.0	26.2	22.3	6.8	8.2	10.0	7.6	10.3 (60)	9.3
3,4,6-Me_3 Gal*p*	2-Gal*p*	1.6	0.6	1.5	0.8	1.2	1.4	2.3	1.0	1.0	1.3	1.4	2.7	1.4	1.0	0.7	1.3 (43)	1.3
2,4,6-Me_3 Gal*p*	3-Gal*p*	1.4	0.8	1.6	1.3	2.0	1.8	2.9	1.5	1.3	1.6	1.8	2.0	2.0	1.9	1.7	1.7 (28)	1.7
2,3,4-Me_3 Gal*p*	6-Gal*p*	1.0	0.6	0.8	0.9	1.2	1.2	1.3	1.9	2.2	1.1	1.1	1.6	1.6	1.0	0.7	1.2 (36)	1.1
2,6-Me_2 Gal*p*	3,4-Gal*p*	0.4	0.1	0.2	0.4	0.4	tr	0.3	0.3	0.4	0.5	0.4	0.3	0.3	0.2	0.2	0.3 (35)	0.3
4,6-Me_2 Gal*p*	2,3-Gal*p*	0.4	0.2	0.2	0.4	0.5	0.4	0.4	0.5	0.5	0.4	0.2	0.5	0.7	0.9	1.0	0.5 (47)	0.4
3,6-Me_2 Gal*p*	2,4-Gal*p*	tr	tr	tr	0.4	0.4	tr	0.4	tr	tr	tr	0.8	-	0.5	0.5	0.5	0.5 (48)	0.5
2,3-Me_2 Gal*p*	4,6-Gal*p*	0.3	tr	0.3	0.5	0.4	0.3	0.3	0.4	0.3	0.5	0.4	tr	0.4	0.5	0.6	0.4 (24)	0.4
3,4-Me_2 Gal*p*	2,6-Gal*p*	0.4	0.2	0.2	0.2	0.6	0.4	0.3	0.3	0.3	0.3	0.4	1.1	0.5	0.3	tr	0.4 (62)	0.3
2,4-Me_2 Gal*p*	3,6-Gal*p*	0.5	0.2	0.3	0.4	0.4	0.4	0.4	0.7	0.7	0.5	0.4	0.4	0.6	1.6	2.1	0.6 (81)	0.4
2-Me Gal*p*	3,4,6-Gal*p*	-	0.5	0.2	0.7	0.3	0.5	0.4	0.6	0.5	0.8	0.4	0.9	0.6	1.0	1.1	0.6 (52)	0.5
4-Me Gal*p*	2,3,6-Gal*p*	0.3	0.3	0.2	0.5	0.4	0.4	0.4	0.7	0.7	0.4	tr	0.9	0.3	0.4	0.3	0.4 (44)	0.4
Gal*p*	2,3,4,6- Gal*p*	8.8	11.4	6.1	12.2	2.8	6.3	1.3	3.2	2.5	0.8	6.7	1.8	0.7	7.1	12.5	5.6 (74)	6.1
2,3,4,6-Me_4 Glc*p*	T-Glc*p*	3.3	3.0	4.1	1.9	5.1	4.0	5.3	4.8	5.0	4.1	2.6	7.3	5.1	2.8	2.1	4.0 (36)	4.1
3,4,6-Me_3 Glc*p*	2-Glc*p*	2.6	1.8	2.1	1.6	2.9	3.0	3.4	3.7	3.7	3.3	2.3	3.5	3.7	2.5	1.8	2.8 (27)	2.9
2,3,4-Me_3 Glc*p*	6-Glc*p*	1.8	1.7	1.4	1.6	1.9	2.4	1.6	2.3	2.4	3.2	2.1	3.1	2.6	2.3	1.7	2.1 (25)	2.1
2,6-Me_2 Glc*p*	3,4-Glc*p*	1.3	0.8	0.8	1.6	1.7	1.6	1.7	2.1	1.7	2.2	1.6	2.0	2.2	2.8	3.2	1.8 (36)	1.7
4,6-Me_2 Glc*p*	2,3-Glc*p*	0.5	0.3	0.4	0.5	0.6	0.6	0.6	0.6	0.5	0.8	0.5	0.7	0.7	0.5	0.6	0.6 (24)	0.6
3,6-Me_2 Glc*p*	2,4-Glc*p*	0.9	0.5	0.5	0.4	1.2	0.8	0.9	1.0	1.0	1.1	0.6	2.7	1.1	0.7	0.6	0.9 (59)	0.9
2,3-Me_2 Glc*p*	4,6-Glc*p*	1.0	0.8	1.2	1.1	1.5	1.4	1.7	2.0	1.4	2.6	1.9	2.7	1.6	2.1	1.3	1.6 (34)	1.5
2,4-Me_2 Glc*p*	3,6-Glc*p*	0.3	0.3	0.3	0.4	0.5	0.5	0.6	0.7	0.4	0.9	0.4	0.6	0.4	1.7	1.3	0.6 (64)	0.5
3,4-Me_2 Glc*p*	2,6-Glc*p*	0.7	0.5	0.4	0.4	0.4	0.5	0.6	0.6	0.6	0.8	0.7	0.8	0.5	0.7	0.5	0.6 (24)	0.6
6-Me Glc*p*	2,3,4-Glc*p*	1.2	0.9	0.7	1.5	1.4	1.3	1.3	1.5	1.4	1.4	1.4	2.1	1.5	2.5	3.2	1.6 (41)	1.4
2-Me Glc*p*	3,4,6-Glc*p*	0.7	0.5	0.3	1.0	0.6	0.9	0.7	0.8	0.7	0.7	1.0	0.9	0.8	1.2	1.1	0.8 (28)	0.8
4-Me Glc*p*	2,3,6-Glc*p*	0.7	0.6	0.3	0.6	0.3	0.2	0.2	0.3	0.2	0.3	0.3	0.2	0.5	0.8	1.2	0.4 (64)	0.3
3-MeGlc*p*	2,4,6-Glc*p*	0.5	0.5	0.3	0.9	0.4	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.5	0.6	0.4 (44)	0.3
Glc*p*	2,3,4,6- Glc*p*	5.3	8.8	4.0	9.6	3.0	4.9	1.2	3.2	4.6	3.3	5.9	2.9	2.1	5.8	8.1	4.8 (51)	4.6

[a] e.g. 2,3,4-Me_3 Rha*p* represents 2,3,4-tri-*O*-methyl-1,5-di-*O*-acetyl rhamnitol

[b] Relative standard deviation

Gal*p* and 2,3,6-substituted Gal*p* were also identified in a polysaccharide of a brown algae (Karmakar *et al.*, 2010; Lee *et al.*, 2011). Residues T-Man*p*, 4-linked Man*p*, T-Glc*p*, 3,4-substituted Glc*p*, and 4,6-sustituted Glc*p* with a sulfate in C-6, were previously identified in red algae polysaccharides, such as a sulfated glucan (Lechat *et al.*, 2000). Among glucose residues, 2,3,4,6-substituted Glc*p* presented the highest mol%. As this is a very complex matrix, it cannot be discarded the hypothesis of the presence of highly substituted Glc, although not yet reported in literature. When a methylation of the polysaccharides was followed by dichloromethane extraction, only few methylated sugars, in very low amount, were observed in the chromatogram, the majority appearing as terminally-linked residues of Xyl*p*, Fuc*p*, Glc*p*, Man*p*, and Gal*p*, as well as 1,4-linked Gal*p* (data not shown). This experiment allowed to infer the occurrence of highly charged polysaccharides, which is in accordance with the highly substituted sugar residues observed when the polysaccharides were methylated and recovered by dialysis. Some of the mannan residues found in polysaccharides from sea salt, namely T-Man*p*, 3,6-substituted Man*p*, 3,4,6-substituted Man*p*, 2,3,6-substituted Man*p* and 2,3,4,6-substituted Man*p*, were previously found in sulfated mannans of red algae (Recalde *et al.*, 2009). Arabinose residues can also arise from algae (Nishide *et al.*, 1990; Recalde *et al.*, 2009; Na *et al.*, 2010). Overall, more than 60% of methylated derivatives found in polysaccharides from sea salt have been already identified in sulfated polysaccharides of algae. These could be released into the medium by cell lysis, apoptosis or exudation (Innamorati, 1995; Giani *et al.*, 2005; Urbani *et al.*, 2005; Engel & Händel, 2011).

Although not reported in **Table 3.4**, since the methodology of carboxyl-reduction used only allowed a qualitative analysis, terminally linked Gal*p*A, terminally linked Glc*p*A and 4-linked Glc*p*A were identified in all the samples analysed. These were also identified in algae polysaccharides, such as ulvan, the major water-soluble polysaccharide found in Ulvales green seaweed (Jiao *et al.*, 2011), in a heteroglycuronan also from a green alga (Ray, 2006), and fucoidans from several species of brown seaweed (Nishide *et al.*, 1990; Li *et al.*, 2008).

Sulfate content of algae polysaccharides can vary between 2% (w/w) (Villanueva *et al.*, 2010) and 45% (w/w) (Navarro *et al.*, 2007). This comprises the sulfate content found in polysaccharides from sea salt (median of 29%, w/w).

Some of the methylated derivatives identified in polysaccharides from sea salt were also found in marine invertebrates. Residues 3,4-substituted Fuc*p*, 2,3-substituted Fuc*p* and 2,3,4-substituted Fuc*p* were previously identified in sulfated fucans of sea urchins (Mulloy *et al.*, 1994; Alves *et al.*, 1997; Mourão & Pereira, 1999), and 3-substituted Fuc*p*, 2,3-substituted Fuc*p* and 2,3,4-substituted Fuc*p* in sulfated fucans of sea cucumber (Mulloy *et al.*, 1994). For these fucans, sulfate was found in C-2 and C-4 of 2,3,4-substituted Fuc*p* (disulfated residue), in C-4 of 3,4-substituted Fuc*p*, and in C-2 of 2,3-substituted Fuc*p*. It is also possible to find sulfated galactans in sea urchins, for which the residue 2,3-substituted Gal*p* with a sulfate in C-2 was identified (Mulloy *et al.*, 1994; Alves *et al.*, 1997). Marine invertebrates ascidians also contain sulfated galactans, being the residue T-Gal*p*, and 3,4-substituted Gal*p* with a sulfate in C-3, identified as part of their structure (Santos *et al.*, 1992). No references were found regarding the chemical structure of sulfated polysaccharides reported for microorganisms living in saltpans.

Quantitatively, there is a mach between sugar and methylation analyses, since they arrive to similar mol% for the neutral sugars identified. However, for the polymeric material samples from sea salt, the sulfate content determined by turbidimetry exceeds in 6 mol% the median of possible positions of binding in sugars. This excess can also have the contribution of the sulfate coming from 3,6-anhydrogalactose of algae carrageenans that are potentially present in sea salt, and which the content was not determined. A good reproducibility was obtained when considering all sea salt samples analysed. Although a natural and heterogeneous product, sea salt seems to have a typical polysaccharide composition.

The presence of highly sulfated polysaccharides in PM from sea salt, as mentioned before, can be explained by their high solubility in water, due to their charge abundance. These structural features may lead to their co-precipitation only when the brine is saturated, forming an ionic network with sea salt cations. These polysaccharide salts may have resist to the action of environment living organism glycosidic enzymes. It is possible that, under these conditions, the sulfated moieties should prevent the action of the enzymes, thus promoting their concentration in the sea salt matrix.

Besides the contribution in the differentiation of sea salt from industrial salt, the presence of polysaccharides salts in sea salt, and its deeper characterisation, may lead to the establishment of potential authenticity markers, i.e. of qualitative and quantitative

characteristics of polysaccharides that could connect the sea salt in study with its saltpan surrounding environment, and potentially with its saltpan of origin.

III.C.5. CONCLUDING REMARKS

This study allowed the isolation, for the first time, of polymeric material from sea salt in amounts that allowed its characterisation. The median amount of polymeric material was 144 mg per kg of salt, e.g. 0.014% (w/w), composed mainly by highly sulfated polysaccharides and a small amount of proteins, as observed by mid-infrared spectroscopy and thermogravimetry. The median sulfated polysaccharide content was estimated to be 461 mg/g of polymeric material, e.g. 46% (w/w), which means 66 mg/kg of sea salt. The median sugar composition of polysaccharides from sea salt was 21 mol% of uronic acid residues, 18 mol% of glucose, 15 mol% of galactose, and 13 mol% of fucose. The sulfate content of polysaccharides from sea salt represented a median of 45 mol%. Glycosidic linkage composition allowed to infer that these polysaccharide salts should have arisen mainly from algae. The knowledge of the occurrence of highly sulfated polysaccharides in quantitative amounts in sea salt opens other paths of research towards a deeper characterisation of biomolecules present in this widespread natural product. Polysaccharides and other biomolecules present in sea salt represent potential organic markers of this natural product, guaranteeing its differentiation and thus contributing to its valuation as a distinct and desirable product. Also, the presence of polysaccharide salts in this matrix opens the possibility of revisiting the sea salt properties in order to explain the possible influence of these compounds in the organoleptic and physico-chemical properties of this product that is largely used in a high number of industries, including those involved in the processing foodstuffs.

III.D. ANALYSIS OF PROTEIN AND TRIACYLGLYCERIDES IN SEA SALT: AN EXPLORATORY STUDY

The present work seeks for the occurrence of protein and triacylglycerides in the organic matter associated to sea salt. With this purpose 16 Atlantic sea salts were analysed. Protein content was attained through amino acid composition of the polymeric material isolated from the sea salts under study, accounting for a median of 35 mg/g of polymeric material. This represented 4.9 mg/kg of dry salt mainly composed by the hydrophobic amino acids alanine (25 mol%), leucine (14), and valine (14). Triacylglycerides, obtained by soxhlet extraction with *n*-hexane, and representing a median content of 1.5 mg/kg of dry salt, were mainly composed by palmitic (42.8 mol%), stearic (13.3), linolenic (12.5), oleic (12.0), and linoleic acid (9.1) residues. The protein and triacylglycerides found in sea salt may arise from the surrounding environment of saltpans, namely from marine organisms such as macro and microalgae, phytoplankton, and cyanobacteria.

III.D.1. AMINO ACIDS COMPOSITION AND PROTEIN CONTENT

Fig. 3.11 shows an example of a GC-FID chromatogram of amino acids from sea salt. The amino acids profile and protein content of polymeric material (PM) from the 16 Atlantic Ocean salts under study are shown in **Table 3.5**. The major amino acid residues (AAs) detected, according to median values, were Ala (25 mol%), Leu (14 mol%), and Val (14 mol%). These AAs were identified in PM of all sea salts under study, as well as Gly, Thr, Ser, and Ile. It was also possible to identify, in some samples, Pro, Asx, Phe, and Glx. The predominance of hydrophobic AAs in the protein associated to sea salt could be related with the increase of the sediment hydrophobicity that occurs along with salt crystals precipitation in the saltpans ponds due to the water evaporation. As a consequence, the sorption capacity towards hydrophobic compounds also increase (Liu & Lee, 2006).

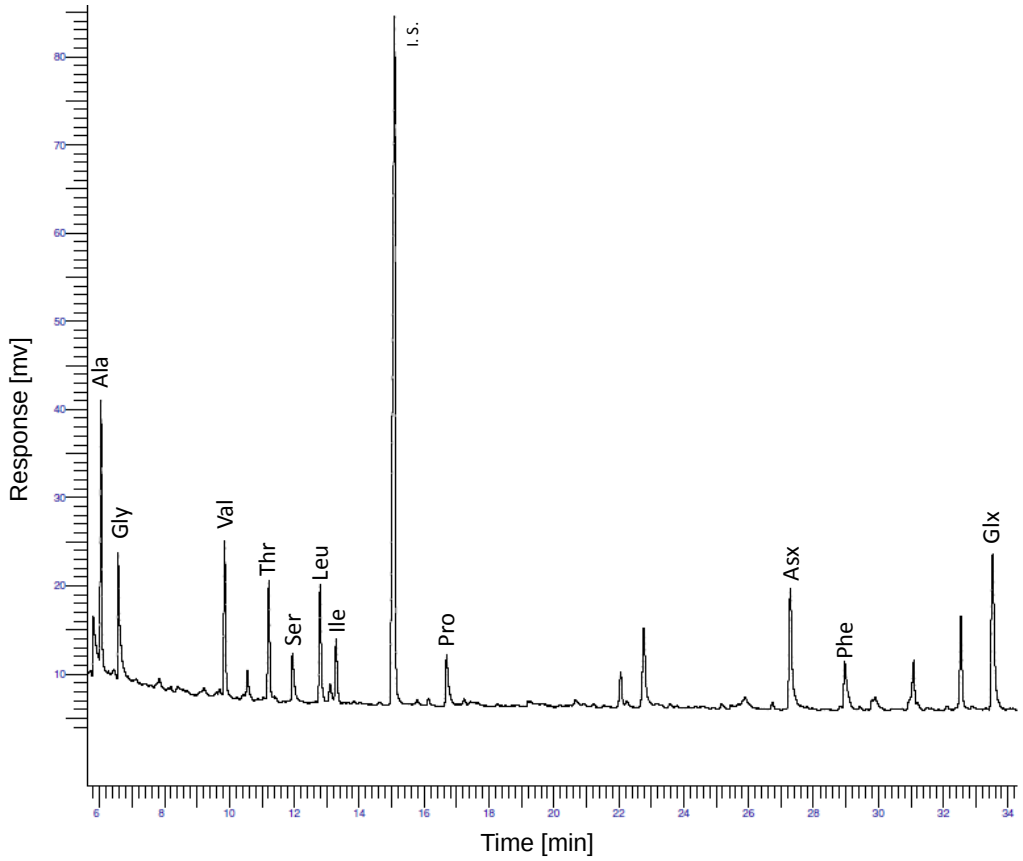

Figure 3.11. GC-FID chromatogram of the amino acid composition of PM from sea salt 18C04. (I.S.- Internal standard)

Based on the AAs composition, the PM from sea salt contains a median protein content of 35 mg/g. This ranged from 23 to 87 mg/g. Consequently, dry sea salt contains a median of 4.9 mg of protein per kg. Average and median values are similar for all the detected AAs. These results justify the protein bands 1640 cm^{-1} C=O stretching (Amide I), and 1550 cm^{-1} C-N stretching and N-H bending (Amide II) detected by mid-infrared spectroscopy, as reported in section III.C.2. Besides protein, another group of compounds containing nitrogen probably present in sea salt are nucleic acids. In spite the amount of deoxyribose and ribose determined in section III.C.4 (**Table 3.3**) for these salts were very low, the presence of nucleic acids could result from DNA and RNA of bacterial communities present in sea salt. As reported by Dufossé *et al.* (2013), it was possible to detect, by a polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis, distinct bacterial communities in sea salts from different geographical areas.

The residues of AAs coming from protein, as constituent of the PM of sea salt, including the more abundant (Ala, Leu, Val), are also constituents of different organisms living in the surrounding marine environment of saltpans, such as macro and microalgae (Barbarino *et al.*, 2005; Gressler *et al.*, 2011), and phytoplankton (Brown & Jeffrey, 1995; Metaxatos *et al.*, 2003). These were also found as dissolved combined AAs in Atlantic marine waters (Sommerville & Preston, 2001).

Table 3.5. Amino acids profile and protein content of the polymeric material of 16 Atlantic Ocean salts.

Sample	Amino acids (mol %)											Protein[a]
	Ala	Gly	Val	Thr	Ser	Leu	Ile	Pro	Asx	Phe	Glx	(mg/g)
PJ04	19	8	16	3	1	15	8	3	12	3	13	60
PJ05	23	8	8	4	4	16	5	3	12	3	14	29
PJ07	35	7	15	4	3	16	9	tr[b]	5	1	5	42
18C04	23	8	14	4	3	11	6	3	10	2	16	34
18C05	22	8	15	3	tr	12	7	3	11	2	15	27
18C07	28	10	14	4	1	14	5	3	8	2	10	36
GCa07	36	8	12	4	3	15	6	2	6	2	6	35
FF04	18	8	14	3	tr	14	8	4	12	2	18	36
FF05	49	11	15	1	tr	12	12	0	0	0	0	36
FF07	33	9	14	4	3	15	7	1	6	2	6	55
CM07	19	7	14	4	2	12	7	4	13	2	17	87
CD07	27	9	5	2	3	17	11	4	6	4	12	37
IR07	21	7	12	5	4	12	8	2	10	2	16	23
LP07	14	5	13	5	5	15	9	3	11	3	18	23
LP09	31	8	12	3	2	13	10	2	9	1	9	25
S07	37	10	12	3	2	13	6	2	6	2	8	26
Average	27 (34)[c]	8 (18)	13 (21)	3 (32)	2 (70)	14 (13)	8 (26)	2 (53)	9 (40)	2 (47)	12 (47)	38 (44)
Median	25	8	14	4	2	14	8	3	9	2	13	35

[a] Expressed in mg per g of polymeric material
[b] Detected in trace amounts
[c] Relative standard deviation (%)

III.D.2. FATTY ACIDS COMPOSITION AND TRIACYLGLYCERIDES CONTENT

The 2 × 20 g of each salt used for the extraction of triacylglycerides (TGs) was insufficient to obtain enough material for a weight quantification of the TGs extracted. For that reason, the content of these compounds were determined based on the fatty acid residues (FAs) individual contribution.

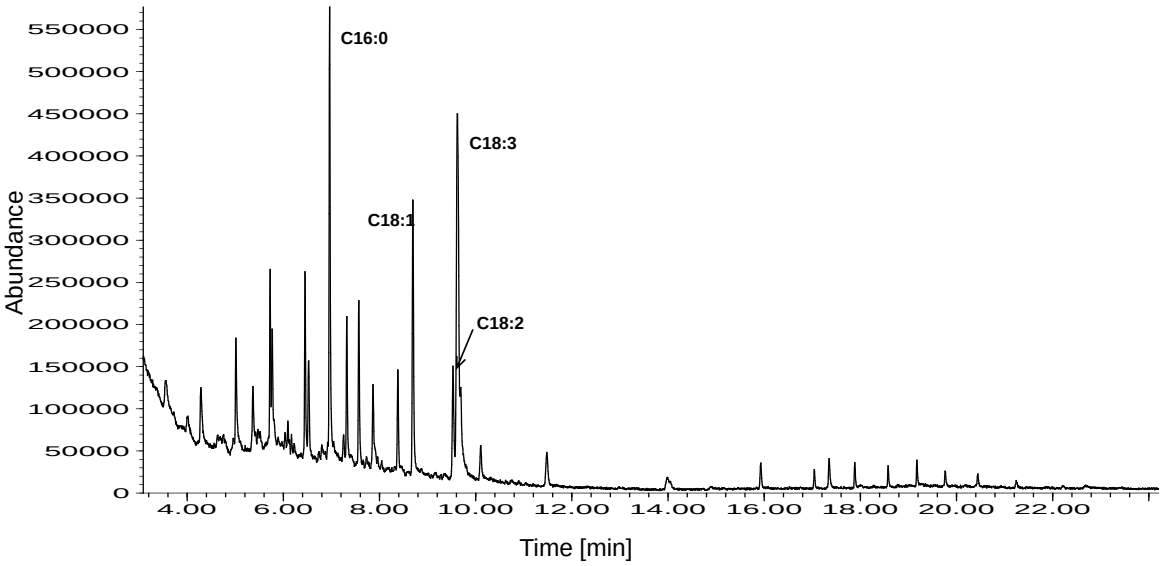

Figure 3.12. GC-qMS chromatogram of the fatty acids composition of sea salt 18C04.

Fig. 3.12 shows an example of a GC-qMS chromatogram of FAs from sea salt. **Table 3.6** shows the FAs profile of the 16 Atlantic Ocean salts under study. The major acid residue detected in TGs, expressed as median, was palmitic acid (C16:0) (42.8 mol%), followed by stearic (C18:0) (13.3 mol%), linolenic (C18:3) (12.5 mol%), oleic (C18:1) (12.0 mol%), and linoleic (C18:2) (9.1 mol%) acids. With exception of C18:3, these FAs were detected in all sea salts. Median values were not so different from the average for most of the FAs identified. Other FAs were also detected, namely myristic (C14:0), pentadecylic (C15:0), arachidic (C20:0), behenic (C22:0), tricosylic (C23:0), lignoceric (C24:0) and cerotic (C26:0) acids. Saturated fatty acids (SFAs) were predominant (56.0 mol%). PUFAs accounted for 28.9 mol% of total fatty acids, and MUFAs accounted for 12.0 mol%. Taking into account the median obtained for the 16 salts it was estimated that the TGs accounted for 1.5 mg per kg of dry sea salt. However, this ranged from 0.2

(GCa07) to 4.8 (18C04) mg per kg of dry sea salt. The relative standard deviation (RSD) values observed for the main FAs of sea salt showed that despite the general trend, there is a significant variability associated to sea salt fatty acid composition.

The TGs found in sea salt may arise from several origins existing in the marine environment. Palmitic acid, the major fatty acid residue identified in sea salt, along with stearic, linolenic, oleic, and linoleic acids, are components of the macroalgal species existing in the Atlantic Ocean (Fleurence *et al.*, 1994; van Ginneken *et al.*, 2011), and also of microalgae and cyanobacteria collected in the Portuguese Atlantic coast (Guedes *et al.*, 2011). These are also components of phytoplankton (Napolitano *et al.*, 1997), and diatoms (Chen, 2012). The fact that palmitic acid is the major fatty acid present in sea salt is in accordance with its occurrence as major fatty acid residue of many marine organisms, such as microalgae and cyanobacteria (Guedes *et al.*, 2011). The majority of the less abundant

Table 3.6. Fatty acids profile and triacylglycerides content of 16 Atlantic Ocean salts.

Sample	Fatty acids (mol%)															Triacylglycerides[a]
	C14:0	C15:0	C16:0	C18:0	C18:1 (ω-9)	C18:2 (ω-6)	C18:3 (ω-3)	C20:0	C22:0	C23:0	C24:0	C26:0	SFAs	MUFAs	PUFAs	(mg/ kg)
PJ04	0.0	1.3	47.6	16.0	8.5	10.1	5.3	1.9	0.0	0.0	6.2	3.2	76.2	8.5	15.4	0.6
PJ05	0.0	0.0	21.8	5.3	16.3	10.7	44.7	0.4	0.3	0.0	0.5	0.0	28.3	16.3	55.4	1.5
PJ07	3.1	1.6	28.8	9.3	10.0	17.0	19.9	1.7	0.0	0.0	5.2	3.4	53.1	10.0	36.9	2.7
18C04	0.0	0.0	11.1	2.9	19.4	9.1	57.1	0.0	0.1	0.0	0.3	0.0	14.4	19.4	66.2	4.8
18C05	0.0	0.0	42.0	13.2	12.0	7.3	25.6	0.0	0.0	0.0	tr[b]	0.0	55.2	12.0	32.9	0.4[c]
18C07	0.0	0.0	38.9	10.8	19.3	13.3	14.1	1.6	0.8	0.0	1.2	0.0	53.3	19.3	27.4	1.4
GCa07	0.0	0.0	57.2	30.4	12.3	tr	0.0	0.0	0.0	0.0	tr	0.0	87.6	12.3	0.0	0.2[c]
FF04	0.0	0.0	61.0	14.6	10.6	5.8	5.5	2.4	0.0	0.0	0.0	0.0	78.0	10.6	11.3	2.2
FF05	0.2	0.1	33.4	8.2	9.6	41.0	3.2	3.2	0.6	0.1	0.3	0.0	46.1	9.6	44.2	n.d.[d]
FF07	3.1	0.0	40.4	10.5	12.7	15.8	15.5	1.6	0.0	0.0	0.4	0.0	56.0	12.7	31.3	0.8
CM07	1.9	0.0	32.0	13.4	9.5	26.8	9.7	4.9	1.6	0.4	0.0	0.0	54.2	9.5	36.5	2.2
CD07	2.0	0.0	54.9	13.6	8.7	3.7	11.1	1.5	0.0	0.0	2.7	1.7	76.4	8.7	14.8	0.5
IR07	1.8	2.3	52.9	26.0	4.8	tr	2.4	1.2	1.8	0.0	6.8	0.0	92.8	4.8	2.4	1.9
LP07	0.0	0.0	43.5	11.7	13.8	9.0	21.3	tr	0.0	0.0	0.7	0.0	55.9	13.8	30.3	0.3[c]
LP09	0.0	0.0	55.5	13.8	11.9	6.7	9.7	0.6	0.8	0.0	0.9	0.0	71.6	11.9	16.4	0.4[c]
S07	0.0	0.0	52.4	13.4	12.2	6.2	13.8	2.0	0.0	0.0	tr	0.0	67.8	12.2	20.0	0.8
Average	0.8 (156)[e]	0.3 (218)	42.1 (33)	13.3 (51)	12.0 (32)	11.4 (90)	16.2 (96)	1.4 (93)	0.4 (158)	0.0 (325)	1.6 (149)	0.5 (225)	60.4 (34)	12.0 (32)	27.6 (66)	1.8 (71)
Median	0.0	0.0	42.8	13.3	12.0	9.1	12.5	1.6	0.0	0.0	0.5	0.0	56.0	12.0	28.9	1.5

[a] Expressed in mg per kg of sea salt dry weight (2-10% of water)
[b] Detected in trace amounts (< 0.05 mol%) by ion extraction
[c] These values were not considered for both average and median, because they represent a very low quantity of triacylglycerides (< 0.5 mg/ kg of sea salt)
[d] Not determined
[e] Relative standard deviation (%)

FAs found in sea salt, such as C24:0, and C20:0 can also arise from these same origins (Chen, 2012; Dawczynski *et al.*, 2007).

Decomposition of unsaturated fatty acids, by means of oxidative and thermal degradation, could give rise to volatile compounds (Min & Smouse, 1985). The decomposition of C18:2 can originate volatile compounds such as heptanal, octanal, and 2-pentyl-furan (Elmore *et al.*, 2004). These three compounds were previously identified in the headspace of some of the sea salts under study produced in 2007 (Section III.A). Also, it was observed that CM07 sample, which according to **Table 3.6** had a content in TGs higher than the median and the highest amount of C18:2 among salts from 2007 harvest, was reported as the sea salt under analysis with the higher GC peak areas for these three volatile compounds (Section III.B). Thus, although a specific study for more conclusive results is needed, it is expected that the TGs present in sea salt, in particular those containing unsaturated fatty acids, contribute to the volatile composition of sea salt.

III.D.3. PCA OF MID-INFRARED SPECTRA OF POLYMERIC MATERIAL FROM SEA SALT

In order to obtain a complementary analysis of the PM from sea salt, regarding its chemical composition, mid-infrared spectroscopy was applied. This allowed the determination of the global composition of the PM of sea salt, as described in section III.C.2. Bands indicating the presence of carbohydrates (3360 cm^{-1} O-H stretching, 2935 cm^{-1} saturated C-H stretching, and 1200 – 850 cm^{-1} stretching of C–O in C–O–H), proteins (1640 cm^{-1} C=O stretching (Amide I), and 1550 cm^{-1} C-N stretching and N-H bending (Amide II)) and sulfate (1380 cm^{-1} ester sulfate, and 1250 cm^{-1} S=O stretching) were detected. Sulfated polysaccharides from sea salt are analysed in detail in section III.C.4. (**Tables 3.3 and 3.4**).

Although seeking for a sea salt global composition profile, the results obtained also showed that the PM composition of the different sea salts under study, had some variability. This variability was estimated by the relative standard deviation (RSD) obtained for the PM components. The content in total sugars presented a RSD of 15, ranging from 13 to 55 for individual sugars (**Table 3.3**), while RSD estimated for sulfate was 33. The RSD of the protein was 16 and varied from 1 to 9 for AAs (**Table 3.5**). Thus,

to evaluate this variability, a PCA was applied to the mid-infrared spectra of the 16 samples of PM from the Atlantic Ocean salts under study.

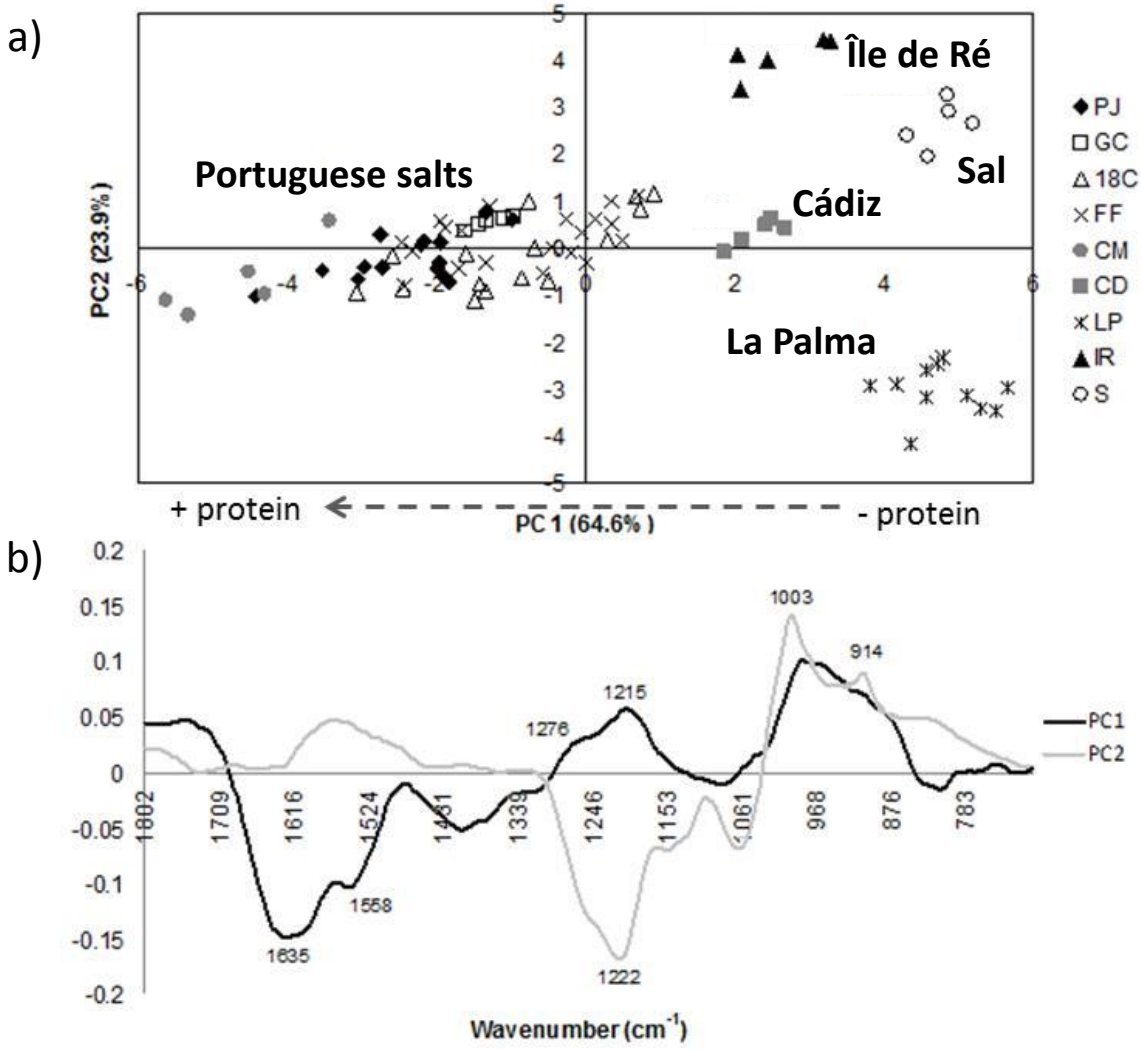

Figure 3.13. PCA analysis based on the mid-infrared spectra (1800-700 cm^{-1}) fingerprint region of polymeric material isolated from 16 Atlantic Ocean salts. (a) scores plot, (b) loadings plot.

The resulting PCA, representing 89% of total variability, shows a separation of sea salts according to their origin (**Fig. 3.13**). The majority of Portuguese samples are grouped in the negative PC1 while all the other salts are placed in the positive PC1 (**Fig. 3.13a**). The non-Portuguese samples are also separated by the PC2 according to their geographical

origins. According to the loadings plot (**Fig. 3.13b**), sea salt distribution along the PC1, representing 65% of the total variability, was related with protein (1635 and 1558 cm^{-1}), and with the variability of a band at 983 cm^{-1}, possibly related with sulfate (Wang *et al.*, 2005). The results of the protein content obtained for the PM of each sea salt under study are in accordance with the sea salt distribution along the PC1, since there is a tendency for the Portuguese samples to present higher amounts of protein. For PC2, representing 24% of total variability, the distribution of the samples seems to be mainly related with sulfate (1222 cm^{-1}) and with a band at 1003 cm^{-1} possibly related with the presence of carrageenans (Sekkal & Legrand, 1993). However, there is not a total match between the positions of the samples under study along the PC2 and their content in sulfate. The content in polysaccharides (850-1200 cm^{-1}) does not influence significantly the variability between samples. These results show that other compounds, with the same functional groups, and therefore contributing for the same bands, are influencing the separation of the sea salt PM in the PCA (**Fig. 3.13**).

III.D.4. CONCLUDING REMARKS

The present study shows that sea salt contains protein and triacylglycerides as part of the organic matter associated to this matrix. The analysis of 16 Atlantic Ocean salts revealed a median content in protein of 35 mg/g of PM, i.e. 4.9 mg/kg of sea salt, mainly composed by the hydrophobic amino acids alanine (25 mol%), leucine (14 mol%), and valine (14 mol%). The median of triacylglycerides content was estimated as 1.5 mg per kg of salt, on a dry basis, presenting as main constituents saturated fatty acids (palmitic and stearic), but also containing unsaturated ones, such as linolenic, oleic, and linoleic. These biomolecules may arise from marine organisms such as macro and microalgae, phytoplankton and cyanobacteria.

Reviewing all previous studies on the organic matter associated to sea salt until now, it is possible to report that this is formed by a volatile fraction, comprising a wide range of compounds such as hydrocarbons, ketones, terpenic compounds and norisoprenoids (Silva *et al.*, 2009; Silva *et al.*, 2010a, Silva *et al.*, 2010b), and by a non-volatile fraction composed by highly sulfated polysaccharides, rich in uronic acids, glucose,

galactose and fucose, by protein, consisting mainly of hydrophobic amino acids Ala, Val and Leu, and by triacylglycerides, with palmitic acid representing the major fatty acid. All these organic components seem to have origin in the surrounding marine environment of saltpans.

In this PhD thesis, a deeper knowledge concerning volatile composition of sea salt was obtained by developing a HS-SPME/GC×GC–ToFMS methodology. Contour plot chromatographic analysis revealed the complexity of marine salt volatile composition and confirmed the importance of a high chromatographic resolution and sensitive technique based on GC×GC–ToFMS. The structured 2D chromatographic profile arising from ^{1}D volatility and ^{2}D polarity was demonstrated, allowing more reliable identifications. From the 157 compounds detected in Aveiro sea salt, furans, haloalkanes, and ethers were identified for the first time. Results obtained for analysis of salt from two diverse locations and harvests over three years suggest loss of volatile compounds according to the salt storage time. These HS-SPME/GC×GC–ToFMS methodology was also applied to Atlantic Ocean salts of seven geographical origins, all produced in the same year. This allowed to identify ten common compounds, among the 165 detected, namely 6-methyl-5-hepten-2-one, 2,2,6-trimethylcyclohexanone, isophorone, ketoisophorone, β-ionone-5,6-epoxide, dihydroactinidiolide, 6,10,14-trimethyl-2-pentadecanone, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate, 2,4,4-trimethylpentane-1,3-diyl bis(2-methylpropanoate), and 2-ethyl-1-hexanol. With exception of the last three, these compounds were classified as carotenoid-derived compounds, which seem to arise mainly from plants or/and algae characteristic of marine environments. These potential volatile markers seem to characterise the sea salt, independently from saltpan, geographic origin, and harvest.

The present PhD work also allowed the isolation and characterisation, for the first time, of polymeric material (PM) from sea salt. A dialysis-based methodology was developed to isolate the PM from sea salt in amounts that allowed its characterisation. The median content of PM isolated from 16 Atlantic Ocean salts was 144 mg per kg of salt, e.g. 0.014% (w/w). Mid-infrared spectroscopy and thermogravimetry revealed a main occurrence of sulfated polysaccharides, and a small amount of proteins in the PM from sea salt. Polysaccharides from sea salt were found to be rich in uronic acid residues (21 mol%), glucose (18 mol%), galactose (15 mol%) and fucose (13 mol%). The median sulfated polysaccharide content was estimated to be 461 mg/g of PM, e.g. 46% (w/w), which accounted for 66 mg/kg of sea salt. Sulfate content represented a median of one sulfate per two sugar residues (45 mol%). Glycosidic linkage composition indicates that the main sugar residues that could carry one or more sulfate residues were identified as fucose and galactose. This allowed to propose that sea salt polysaccharides arise mainly, due to their

abundance in marine environments, from algae. Another important group of biomolecules also found in sea salt were proteins. The amino acid profile of the polymeric material from sea salt presented, as the most abundant, the hydrophobic amino acids Ala (25 mol%), Leu (14), and Val (14), being the median protein content of PM from the 16 Atlantic Ocean salts, 35 mg/g, i.e. 4.9 mg/kg of sea salt.

Beside the occurrence of hydrophobic volatile compounds in sea salt, hydrophobic non volatile compounds were also detected. Triacylglycerides were obtained from sea salt by soxhlet extraction with *n*-hexane. Fatty acid composition revealed as major acid residues, in median, palmitic (43 mol%), followed by stearic (13 mol%), linolenic (12 mol%), oleic (12 mol%), and linoleic (9 mol%) acids. SFAs represented the major fraction (56 mol%) followed by PUFAs (29 mol%), and MUFAs (12 mol%). The median triacylglycerides content obtained for the 16 Atlantic Ocean salts was 1.5 mg/kg of sea salt. Both protein and triacylglycerides seem to arise from macro and microalgae, phytoplankton, and cyanobacteria.

In summary, beyond sodium chloride and other minerals, sea salt is also composed by organic compounds. This organic matter is characterised by:

1. a volatile fraction, comprising a wide range of compounds such as hydrocarbons, ketones, terpenic compounds, and norisoprenoids, and by a non-volatile fraction,
2. sulfated polysaccharides, rich in uronic acid residues, glucose, galactose, and fucose,
3. protein, consisting mainly of hydrophobic amino acids (Ala, Val, and Leu),
4. triacylglycerides, with palmitic acid representing the major fatty acid residues.

All these organic components seem to be related with the surrounding marine environment of saltpans, arising mainly from algae, phytoplankton, bacterial communities, and anthropogenic activity.

Future Work

The establishment that sea salt contains a diverse number and type of volatile compounds, highly sulfated polysaccharides, proteins, and triacylglycerides allows the rise of several questions:

- What is the biological origin of these compounds?
- What are the species that contribute more for the sea salt organic compounds?
- What is the contribution of the environment and anthropogenic activities to the sea salt organic compound composition?
- Are these compounds also present in the sea salts from other oceans?
- Should these compounds influence the organoleptic and physico-chemical properties of the foodstuffs where sea salt is included, acting as potential sea salt taste modulators?

These are questions that should be answered in the near future.

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